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Study protocol for a 9-month randomised controlled trial assessing the effects of almonds vs. carbohydrate-rich snack foods on weight loss and weight maintenance.

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Strengths and limitations of the study:

- To our knowledge, this will be the first randomised controlled trial to assess whether the inclusion of almonds vs. carbohydrate-rich snack foods in an otherwise nut-free diet will improve weight loss and limit weight regain.
- A wide range of outcomes will be assessed including but not limited to; body composition, resting and total daily energy expenditure, appetite regulation, cardiometabolic health, liver health, inflammatory markers and effects on the gut microbiome.
- Both objective and subjective appetite regulation will be evaluated, adding to our current limited knowledge of the effects of almonds on appetite control.
- The metagenomic analysis that will be performed will be a substantial advance on our current understanding of the impact of almonds on the gut microbiome (previously limited to amplicon sequencing approaches).
- A potential limitation of this study is that it will only be feasible to follow participants for 6 months after initial weight loss.

ABSTRACT

Introduction Epidemiological studies indicate an inverse association between nut consumption and body mass index (BMI). However, clinical trials evaluating the effects of nut consumption compared to a nut-free diet on adiposity have reported mixed findings with some studies reporting greater weight loss and others reporting no weight change. This paper describes the rationale and detailed protocol for a randomised controlled trial assessing whether the inclusion of almonds or carbohydrate-rich snacks in an otherwise nut-free energy-restricted diet will promote weight loss during 3 months of energy restriction and limit weight regain during 6 months of weight maintenance.

Methods and analysis One hundred and thirty-four adults aged 25-65 years with a BMI of 27.5-34.9kg/m^2 will be recruited and randomly allocated to either the almond-enriched diet (AED) [15% energy from almonds] or a nut-free control diet (NFD) [15% energy from carbohydrate-rich snack foods]. Weight loss will be achieved through a 30% energy restriction over 3 months, and weight maintenance will be encouraged for 6 months by increasing overall energy intake by ~120-180 kcal/day as required. Body composition, resting energy expenditure, total daily energy expenditure (via doubly labelled water), physical activity, appetite regulation, cardiometabolic health, gut microbiome, liver health, inflammatory factors, eating behaviours, mood and personality, functional mobility and pain, quality of life and sleep patterns will be measured throughout the 9-month trial. The effects of intervention on the outcome measures over time will be analysed using random effects mixed models, with treatment (AED or NFD) and time (baseline, 3 months, and 9 months) being the between and within factors respectively in the analysis.

Ethics and dissemination Ethics approval was obtained from the University of South Australia Human Research Ethics Committee (201436). Results from this trial will be disseminated through publication in peer-reviewed journals, national and international presentations.

Trial registration number ACTRN12618001861246; Pre-results.

INTRODUCTION

Epidemiological studies report associations between increased frequency of nut consumption and lower body weight (1-4). This is supported by clinical data which suggests that regular nut intake has a positive impact on adiposity, insulin resistance and related metabolic abnormalities (5-11). Despite this, many people still avoid eating nuts due to the perception that they lead to weight gain based on their high energy and fat content. Nut consumption in many countries including Australia and America is low, (6 g/day and 3.3 g/day per capita, respectively) (12-14) with the prevalence of adult nut consumers being ~ 16-20% in Australia (15) and America (16). Such low consumption suggests there is scope to increase consumption in both countries.

Data from the National Health and Nutrition Examination Survey indicated that nut consumption was associated with a lower body mass index (BMI) (27.7 ± 0.2 kg/m^2 vs 28.1 ± 0.1 kg/m^2, $p < 0.05$) and waist circumference (95.6 ± 0.4 cm vs 96.4 ± 0.3 cm, $p < 0.05$), and tree nut consumers had lower body weight than non-consumers (78.8 ± 0.7 kg vs 80.7 ± 0.3 kg, $p < 0.05$) (16). When considering almonds specifically, randomised controlled trials have reported greater weight loss (17, 18) or improved body composition (reduced total fat and truncal fat) (19, 20) on hypocaloric diets with the inclusion of almonds compared to a nut-free diet. However, recent meta-analyses of clinical trials evaluating the effects of nut consumption on adiposity have reported no difference in body weight, BMI or waist circumference when comparing diets including nuts against control diets (5, 9). Despite these mixed findings, changes in body fat distribution and reductions in fat stored in the liver can improve metabolic outcomes independent of weight changes. Inclusion of nuts may help prevent and manage non-alcoholic fatty liver disease (NAFLD) (21, 22) and almond consumption has been associated with reductions in circulating liver enzyme concentrations (23). Additionally, data from large cohort studies suggest that frequent nut consumption may lower the risk of weight gain, with consumption on 5 or more days per week showing the greatest effect (24, 25). Similar findings have also been reported from the EPIC-PANACEA cohort (4). Prospective analysis of cohorts of healthy adults show that the average weight gain over 4 years was 3.3 lb (~1.5 kg). This weight gain was inversely associated with nut consumption (26).

Weight regain following initial weight loss is common and contributes, in part, to the obesity epidemic (27). Randomised controlled trials assessing weight loss

maintenance report a 30-35% weight regain in the first year following a weight loss intervention with a 76% weight regain at 4 years post-treatment (28). Weight regain suggests there is a need for nutritional strategies that prevent weight regain. Therefore assessing the effects of nutritional strategies, such as the inclusion of nuts for the prevention of weight regain, is paramount. Diets high in protein appear to limit weight regain (29) with higher intakes of non-cereal plant proteins, such as in nuts, associated with a protective (30). Nuts are rich in protein, fibre and monounsaturated fat, which have been suggested to contribute to their positive effect on appetite regulation (31, 32) and almonds, specifically, have been shown to have positive effects on subjective ratings of appetite (33, 34). However, the majority of assessments of appetite regulation with nuts have been conducted acutely and subjectively, and few have assessed appetite hormones (35). Reduced food cravings have been associated with long-term weight loss success (36); thus, assessing both of these outcomes following regular almond consumption is important.

Previous studies have reported significant differences in gut microbiota between lean and obese individuals (37) and modulation at the phylum and genus levels following weight loss (38). Changes in gut microbiota have also been observed following consumption of diets containing almonds (39) and almond skins (40), although not consistently in all populations (41). Significant increases in the relative abundance of bacterial taxa comprising of Ruminiclostridium, and members of the Ruminococcaceae and Lachnospiraceae families, under trial conditions are implicated in the degradation of complex dietary plant-derived polysaccharides, and the production of beneficial short-chain fatty acids (42). It has been suggested that bacterial products may impact on short-term intestinal satiety pathways and long-term appetite control, acting directly on hypothalamic neurons (43). However, it is unclear whether the changes in gut microbiota associated with almond consumption may help limit weight regain and the relationship with other biomarkers of cardiometabolic risk.

OBJECTIVES

Primary objective

The primary aim of this project is to evaluate whether inclusion of 15% of energy from almonds or carbohydrate-rich snacks in an otherwise nut-free energy-restricted

diet, will improve weight loss during 3 months of dietary energy restriction and limit weight regain during 6 months of weight maintenance. We hypothesise that consuming almonds as 15% of energy requirements compared with a nut-free diet will lead to greater weight loss during the energy restriction phase of 3 months and limit weight regain during the weight maintenance period of 6 months.

Secondary objectives

The secondary aim is to evaluate whether an almond or nut-free diet improves body composition and body fat distribution (reduced waist circumference and abdominal fat depots), impacts resting and total daily energy expenditure and improves subjective and objective measures of satiety.

Tertiary objectives

The tertiary aim is to evaluate whether an almond or nut-free diet reduces fat accumulation in the liver and improves liver enzyme profiles, results in beneficial changes in the composition of the gut microbiome, improves inflammatory biomarkers and cardiometabolic health outcomes including blood lipid profiles, glucose and insulin, and improves self-reported eating behaviours, mood, personality, pain, functional mobility, quality of life, sleep and physical activity patterns.

METHODS AND ANALYSIS

Study design

The study is designed as a 9-month randomised controlled parallel-arm dietary intervention. The study will be conducted in the research facilities of the Alliance for Research in Exercise, Nutrition and Activity (ARENA) at the University of South Australia, Adelaide. The SPIRIT guidelines were used in the development of this protocol (44).

Patient and public involvement

Development of this research protocol was done without patient involvement. The final study results will be disseminated to all participants.

Participants

Eligibility criteria

Participants will be male and female volunteers, aged 25-65 years, with a BMI of 27.5-34.9 kg/m^2. Participants will be non-smokers (minimum 6 months) and weight stable (within 5 kg) for 3 months prior to enrolment.

Exclusion criteria

Participants will be excluded if they have cardiovascular disease, Type 1 or Type 2 diabetes, kidney or liver disease, or any gastrointestinal disorders requiring medical nutrition therapy (e.g., Crohn's, Irritable Bowel, Coeliac Disease). Further restrictions include the use of any vitamin, mineral, herbal supplement or medication that may impact on study outcomes or has changed in the last 3 months. In addition, participants will be excluded if they have an allergy to nuts, gluten or other components of the test foods or are unable to chew hard foods such as nuts or have consumed more than 30 g of nuts per week in the month prior to beginning the trial or consume more than 14 standard drinks per week. Further restrictions include pregnancy or breastfeeding, fear of enclosed spaces (claustrophobia), or an unwillingness to be randomised to either experimental group.

Recruitment/screening

Participants will be recruited from the public through radio, TV, printed media, internet-based advertisements and flyer distribution. Procedures will occur in accordance with ethical standards, including obtaining written informed consent.

Interested participants will be sent the participant information and a diet and lifestyle questionnaire (DLQ) to determine eligibility. Participants who appear eligible from DLQ responses will be assigned a screening number and will undergo an initial screening interview over the telephone to review medical history, concomitant medication and supplementation. Prospective participants will attend the clinical research facility approximately 3 weeks before baseline where eligibility will be confirmed, and the likelihood to consume test foods will be established via liking and palatability questionnaires. Participants who meet the required criteria and are deemed eligible, will be asked to provide written informed consent in the presence of

the investigator and will proceed with a pre-baseline clinic appointment, 2 weeks prior to baseline.

Randomisation, allocation concealment and sequence generation

Data collected at the screening visit will be used to assign participants to groups based on age, sex, and BMI in the process of randomisation by minimization (45). Minimisation will ensure baseline characteristics are balanced between the treatment groups and has been proposed to be the most suitable randomisation method for small clinical trials, such as the proposed study, to reduce bias (45, 46). A staff member independent of the study outcome assessments and statistical analysis will perform the treatment allocation and maintain the randomisation list in a secure location with access limited to authorised personnel. As the participants are consuming whole foods, which are easily identified, the participants and staff involved in diet management cannot be blinded. Staff conducting clinical assessments at baseline, 3 months and 9 months will remain blinded to treatment group allocation. Participants will be asked not to disclose the foods they are consuming to the researchers. Researchers conducting assessments and analysing data will remain blinded until the completion of statistical analysis. Decoding procedures will not be necessary during the study because the participants will know which foods they are consuming.

Sample size calculation

One hundred participants will provide 80% power to detect a 2.4 kg difference in weight loss (18) and a 1.7 kg difference in weight regain (based on variance in our pilot data) (Wilson AL et al. Nudging Weight Loss Maintenance in Adults with Type-2 Diabetes: A Pilot Intervention) between the two groups (α-level of 0.05). One hundred and thirty-four participants will be recruited (n=67 in each group) to allow for a 25% dropout.

Pre-intervention

Two weeks prior to baseline visits participants will be asked to attend a pre-baseline session. During this visit, a flash blood glucose monitoring sensor (FreeStyle Libre,

Abbott, Australia) will be inserted for collection of blood glucose measures for 2 weeks, a wrist-worn accelerometer (GENEActiv, Activinsights Ltd, UK) will be provided for measuring physical activity and sleep patterns for 2 weeks, and a test kit will be provided for a one-off stool sample collection. Participants will be asked to keep a 4-day weighed food diary (non-consecutive days with 1 weekend day) and a 14-day sleep diary. Several questionnaires will also be administered to assess eating behaviour, mood, stress and personality (see Table 1).

Table 1. Outcome Measures at Each Time Point

	Study Period									
	Screening		Enrolment		Allocation		Post-allocation		Close-out	
Time (Weeks) from Start of Dietary Intervention	**-3**	**-2**	**-1 (D1)**	**0 (D2)**	**10**	**12 (D1)**	**13 (D2)**	**35**	**36 (D1)**	**37 (D2)**
Study Food Liking & Palatability Scores										
Labelled Affect Magnitude Scale	X								X	
Food Action Rating Scale	X								X	
Body Composition										
Height	X	X								
Weight (& Body Mass Index)	X	X	X	X	X	X	X	X	X	X
Total and Truncal Fat Mass (DXA)			X			X			X	
Total and Truncal Fat-Free Mass (DXA)			X			X			X	
Visceral Adipose Tissue (DXA)			X			X			X	
Waist Circumference			X			X			X	
Energy Expenditure										
Accelerometry		X			X			X		
Resting Energy Expenditure (Indirect Calorimetry)			X			X			X	
Total Daily Energy Expenditure (Doubly Labelled Water)[a]			X			X			X	
International Physical Activity Questionnaire			X			X			X	
Appetite Regulation and Eating Behaviour										
Energy Intake										
4-day Food Diary		X			X			X		
24-hour Diet Recalls[b]							X			X
Fasting and Postprandial Gut Hormones & Glucose										
Glucagon-like peptide-1				X			X			X
Ghrelin				X			X			X
Leptin				X			X			X
Pancreatic Polypeptide				X			X			X

	Study Period									
	Screening		Enrolment		Allocation		Post-allocation		Close-out	
Time (Weeks) from Start of Dietary Intervention	**-3**	**-2**	**-1 (D1)**	**0 (D2)**	**10**	**12 (D1)**	**13 (D2)**	**35**	**36 (D1)**	**37 (D2)**
Glucose-Dependent Insulinotropic Polypeptide (Gastric Inhibitory Polypeptide)				X			X			X
Peptide Tyrosine Tyrosine (Peptide YY)				X			X			X
C-Peptide				X			X			X
Cholecystokinin (CCK)				X			X			X
Glucagon				X			X			X
Glucose				X			X			X
Drive to Eat and Eating Behaviour										
Subjective Drive to Eat (Visual Analogue Scales) – Fasting and Postprandial				X			X			X
Energy Consumed at a Buffet Meal				X			X			X
Power of Food Survey			X			X			X	
Food Craving Scale			X			X			X	
Fickiness Questionnaire			X						X	
Eating Attitude Test (EAT-26)			X			X			X	
Control of Eating Questionnaire			X			X			X	
Psychology and Health										
General Health, Pain, Mobility, Mood and Personality										
Short-form 36 (SF36) Questionnaire				X			X			X
Profile of Mood States (POMS)				X			X			X
Perceived Stress Scale		X				X			X	
Zung Self-Rating Scale		X				X			X	
McGill Pain Scale and Chronic & Acute Pain Scales (Visual Analogue Scales)				X			X			X
Timed Up and Go (Functional Mobility)			X			X			X	
Eysenck Personality Questionnaire		X								
Brief Sensation Seeking Scale		X								
Gut Health										
Faecal microbiome composition		X			X			X		

	Study Period									
	Screening		Enrolment		Allocation		Post-allocation		Close-out	
Time (Weeks) from Start of Dietary Intervention	**-3**	**-2**	**-1 (D1)**	**0 (D2)**	**10**	**12 (D1)**	**13 (D2)**	**35**	**36 (D1)**	**37 (D2)**
Liver Health										
Liver Ultrasound				X			X			X
Alanine Aminotransferase (ALT)			X			X			X	
Aspartate Aminotransferase (AST)			X			X			X	
Alkaline Phosphatase (ALP)			X			X			X	
γ-Glutamyltransferase (GGT)			X			X			X	
Cardiometabolic Health										
Blood pressure	X	X	X			X			X	
Continuous Glucose Monitoring		X			X			X		
Insulin				X			X			X
HOMA				X			X			X
Triglycerides			X	X		X	X		X	X
Cholesterols (TC, HDL-C, LDL-C, VLDL-C, IDL-C, Oxidised LDL)			X			X	X		X	X
Apolipoprotein B			X			X			X	
Apolipoprotein A1			X			X			X	
Inflammatory Markers										
F2-Isoprostane levels (plasma + urine)			X			X			X	
C-reactive protein			X			X			X	
Adiponectin			X			X			X	
Sleep Patterns										
Pittsburg Sleep Quality Index			X			X			X	
14-day sleep diary		X			X			X		
Biomarkers of Compliance										
α-tocopherol (plasma + urine)			X			X			X	

[a] Sub-sample only; [b] 3 x 24-hour recalls were completed at random intervals between 0-13 weeks and 13-37 weeks; DXA, dual-energy X-ray absorptiometry; HDL-C, high density lipoprotein-cholesterol; IDL-C, intermediate density lipoprotein-cholesterol; LDL-C, low density lipoprotein-cholesterol; TC, total cholesterol; VLDL-C, very low density lipoprotein-cholesterol.

Study Intervention

The intervention will be a 9-month protocol, consisting of 3 months weight loss and 6 months weight maintenance. During the 9-month study period, participants in the almond group (AED) will incorporate 15% of their energy as unsalted, whole, natural almonds with skins while participants in the control group (NFD) will include 15% of their energy consumed as carbohydrate-rich snack foods (oven-baked fruit cereal bar and rice crackers) as part of a 30% energy restricted weight loss diet. It is expected that the minimum quantity of almonds required to contribute 15% of energy will be 30 g, which is consistent with dietary guidelines (47). The control foods have been selected as they are commonly consumed snacks, and do not contain the beneficial micro and macro-nutrients available in almonds but are of an equal energy density. Participants will be provided with test foods to consume 6 days per week so that they have 1 day per week free from consuming test food. This has previously been found to support compliance (48). Checklists will be used to record daily consumption of study food and participants will be asked to return leftover food to calculate compliance scores. The threshold of compliance with test food consumption is >80%. All participants will be asked to avoid all other nuts and nut products during the entire study.

Energy requirements will be determined by using the Schofield Equation, based on age, sex, and initial body weight (49) less approximately 30% to achieve 0.5-1 kg weight loss per week. Participants will be guided to consume a variety of foods within all five food groups to ensure they are still meeting nutrient reference guidelines consistent with Australian Dietary Guidelines (47). Participants will be provided with food group serve advice consistent with an energy restriction plan closest to their weight loss energy requirement of either 1200, 1500 or 1800 kcal/day (5000, 6300 or 7600 kJ/day). Three sample meal plans will be provided, as well as a recommended discretionary serve allowance of 2 per week, consistent with their energy requirement. Diet checklists will be used to assist with dietary compliance. During the weight maintenance phase, participants will be encouraged to stabilise their weight by increasing their overall energy intake by ~120-180 kcal/day (~500-750 kJ/day), as required.

Participants will attend two baseline appointments (Day 1, -1 week and Day 2 – 0 weeks before intervention) and will be asked to refrain from alcohol for 24 hours and

fast for a minimum of 10 hours prior to assessments. Details of tests, assessments and outcome measures completed on Days 1 and 2 are available in **Table 1**. Participants will meet with the study dietitian at the end of the Day 2 appointment to receive initial dietary counselling and instructions on test food consumption requirements. They will then meet individually with a dietitian every 2 weeks during the weight loss phase to have their weight monitored and test food compliance checked. During weight maintenance, participants will meet individually with the dietitian fortnightly for the first month and then monthly in small groups. **Figure 1** outlines the study timeline. Adherence to energy-restricted diets will be assessed using 3 x 24-hour dietary recalls (via phone, at random times) during each phase, with weighed food diaries at baseline and at the end of the weight loss and weight maintenance phases. Participants will also be encouraged to meet national physical activity guidelines for Australian adults - 150 to 300 minutes (2 ½ to 5 hours) of moderate intensity physical activity or equivalent, per week (47). Accelerometer data will allow us to monitor whether 'unexpected' weight change might be explained by physical activity levels, and will assist in understanding the weight loss effects attributable to consuming almonds. Two weeks before the end of the weight loss and weight maintenance phases, participants will repeat testing that occurred at pre-baseline and baseline Day 1 and Day 2 (see Table 1).

Data Collection

The following section outlines the data and biochemical samples being collected during the test periods (see Table 1 for a summary).

Anthropometry

All anthropometric assessments will be conducted with participants barefoot and wearing light clothing. Height will be measured twice to the nearest 1 mm with the average value calculated and recorded using a stadiometer at baseline (SECA 216 Height Measuring Rod, SECA). Body weight will be recorded to the nearest 100 g following an overnight fast and will be measured twice on each occasion using calibrated electronic scales (SECA 703 wireless column scales, SECA) and the average value calculated. The same scales will be used throughout the intervention. BMI will be calculated as weight/height squared (kg/m^2). Waist circumference will be measured, according to the protocol of the International Society for the Advancement

of Kinanthropometry (50), using a metal measuring tape at the narrowest point of the abdomen or, if there is no obvious narrowing, at the midpoint between the lower costal (10th rib) border and the iliac crest. Two measurements will be taken unless they differ by more than 2% whereby a third measurement will be obtained. The mean of the measurements will be used for analysis. Body composition will be determined from a whole body dual-energy x-ray absorptiometry (DXA) scan, (Lunar ProdigyModel, General Electric, Madison WI USA). Participants will wear a light disposable gown, and all external metal objects will be removed. Participants will not be scanned if pregnant. Total body fat mass (%, kg), total body lean mass (%, kg), regional fat mass (arms, legs, trunk and abdominal fat mass, android, gynoid (kg)) and visceral adipose tissue amount and volume (kg and cm^3), will be obtained using enCORE™ 2015 software (GE Healthcare enCORE version 13.31).

In addition to the measurements taken at baseline, at the end of weight loss and weight maintenance phases, weight will also be measured during dietetic counselling appointments to provide feedback to participants. We will also lend participants scales (Withings/Nokia WBS06, Nokia) with Bluetooth capacity to enable them to monitor their weight at least twice per week at home and for these data to be sent to research staff to assist with weight monitoring. Regular weight monitoring has been shown to enhance success in weight loss (51) and weight maintenance trials (52).

Biochemical Measures

At Day 1 baseline, 3-month and 9-month appointments, fasting (>10 hours) venous blood samples will be taken by a trained phlebotomist. Collected blood samples will be centrifuged (4° C, 4000 rpm, 10 mins) to separate plasma or serum and stored at −80°C for later analysis (see Table 2).

Table 2. Blood Analysis

Parameter	Analysis Method	Sample Collected (Additives)
TC	Vertical Auto Profile (VAP II) (53) and proton magnetic resonance spectroscopy (54)	Plasma
HDL-C	Vertical Auto Profile (VAP II) (53) and proton magnetic resonance spectroscopy (54)	Plasma
LDL-C	Vertical Auto Profile (VAP II) (53) and proton magnetic resonance spectroscopy (54)	Plasma
VLDL-C	Vertical Auto Profile (VAP II) (53) and proton magnetic resonance spectroscopy (54)	Plasma
IDL-C	Vertical Auto Profile (VAP II) (53)and proton magnetic resonance spectroscopy (54)	Plasma
Lipoprotein(a)	Vertical Auto Profile (VAP II) (53)and proton magnetic resonance spectroscopy (54)	Plasma
Oxidised LDL-C	Solid phase 2-site ELISA	Plasma
Triglyceride	Konelab Auto Analyser	Plasma
APOB	Vertical Auto Profile (VAP II) (53) and patented equations (20)	Serum
APOA1	Vertical Auto Profile (VAP II) (53) and patented equations (20)	Serum
hs-CRP	Konelab Auto Analyser	Serum
Adiponectin	ELISA	Serum
F2-Isoprostanes	Electron-capture negative-ion gas chromatography-mass spectrometry (55)	Plasma + Urine (Butylated Hydroxytoluene)
α-tocopherol	High-performance liquid chromatography using the photo-diode array method (56)	Plasma + Urine
ALT	Abbott Alinity C	Serum
AST	Abbott Alinity C	Serum
ALP	Abbott Alinity C	Serum
GGT	Abbott Alinity C	Serum
Glucose	Konelab Auto Analyser	Plasma
Insulin	Mercodia ELISA	Plasma (Protease inhibitor and DPP-IV)
HOMA	Calculated using the Homeostasis Model Assessment Calculator v.2.3.3 (57)	-
Glucagon	Multiplex analysis system	Plasma (Protease inhibitor and DPP-IV)
GLP-1	Multiplex analysis system	Plasma (Protease inhibitor and DPP-IV)
Ghrelin	Multiplex analysis system	Plasma (Protease inhibitor and DPP-IV)

Leptin	Multiplex analysis system	Plasma (Protease inhibitor and DPP-IV)
Pancreatic Polypeptide	Multiplex analysis system	Plasma (Protease inhibitor and DPP-IV)
GIP	Multiplex analysis system	Plasma (Protease inhibitor and DPP-IV)
PYY	Multiplex analysis system	Plasma (Protease inhibitor and DPP-IV)
C-Peptide	Multiplex analysis system	Plasma (Protease inhibitor and DPP-IV)
CCK	ELISA (Ray Biotech)	Plasma (Protease inhibitor and DPP-IV)

ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; APOA1, apolipoprotein A1; APOB, apolipoprotein B; CCK, cholecystokinin; GGT, γ-Glutamyltransferase; GLP-1, glucagon-like peptide-1; GIP, glucose-dependent insulinotropic polypeptide (gastric inhibitory polypeptide); HDL-C, high density lipoprotein-cholesterol; hs-CRP, high-sensitivity c-reactive protein; IDL-C, intermediate density lipoprotein-cholesterol; LDL-C, low density lipoprotein-cholesterol; PYY, peptide tyrosine tyrosine (peptide YY); TC, total cholesterol; VLDL-C, very low density lipoprotein-cholesterol.

Lipids

Serum lipid, cholesterol lipoprotein subclasses, and apolipoproteins will be assayed using Vertical Auto Profile (VAP II) (Atherotech Diagnostics Lab, Birmingham, AL), which directly measures cholesterol in all lipoprotein classes (53). Plasma lipoprotein particle number and size will be assessed by a proton magnetic resonance spectroscopy assay (NMR LipoProfile III; LipoScience, Raleigh, NC), which measures the particle concentrations of lipoprotein subclasses and average particle size of lipoproteins (54). This assay quantifies cholesterol concentrations of total lipoprotein, HDL, LDL, very-low-density lipoprotein (VLDL), lipoprotein(a) [Lp(a)], intermediate-density lipoprotein (IDL), and HDL, LDL, VLDL, and IDL subclasses. ApoB and ApoA1 will be calculated using results from the VAP test and patented equations (20). Serum oxidised LDL will be measured in duplicate by a solid phase 2-site ELISA (Mercodia, Uppsala, Sweden). Plasma triglyceride (%CV <5%) will be measured using a Konelab Auto Analyser.

Inflammatory Markers

Fasting plasma and a spot urine sample will be collected for analysis of F2-Isoprostanes as biomarkers of oxidative stress. Samples will be stabilised with butylated hydroxytoluene (BHT). Plasma and urine F2-Isoprostanes will be measured as total (free plus esterified) F2-Isoprostanes using electron-capture negative-ion gas chromatography–mass spectrometry as described previously (55). Serum will be collected for assessment of adiponectin by ELISA (Linco Research, St. Charles, Missouri) (58) and high-sensitivity C-reactive protein (hs-CRP) (%CV intra-assay 2.9%, inter-assay 1.9%) will be measured using a Konelab Auto Analyser.

Liver Enzymes

Fasting serum levels of alanine aminotransferase (ALT) (%CV intra-assay 0.5%, inter-assay 1.0%), aspartate aminotransferase (AST) (%CV intra-assay 0.6%, inter-assay 0.8%), alkaline phosphatase (ALP) (%CV intra-assay 0.3%, inter-assay 1.7%) and γ-glutamyltransferase (GGT) (%CV intra-assay 0.4%, inter-assay 1.1%) and will be measured using a local pathology service (23). To eliminate the effect of freeze-thawing of samples that may lower enzyme activity values, ALT, AST, ALP and GGT testing will be conducted on samples immediately transferred to the pathology laboratory.

Biomarkers of Compliance

Erythrocyte membrane, plasma and urinary α-tocopherol levels will be analysed as measures of compliance of consuming almonds. Samples will be assessed for α-tocopherol using high-performance liquid chromatography with photo-diode array method according to Liu et al. (56).

Appetite Regulation

At Day 2 baseline, 3-month and 9-month, objective and subjective measures of appetite and satiety will be assessed. A test snack will be consumed following an overnight fast (>10 hours). The test snack for the AED group will comprise 15% of daily energy intake from almonds, and the NFD group will have 15% of daily energy intake from a high carbohydrate snack (oven-baked fruit filled bar). Blood samples will be obtained through venipuncture using the BD Nexiva™ cannula blood collection system by a trained phlebotomist at time 0 (before the test snack) and every 30 minutes for 2 hours post the test snack, with participants asked to consume the test snack within 10 minutes. Collected blood samples will be centrifuged (4° C, 4000 rpm, 10 mins) to separate plasma or serum and stored at −80°C for later analysis of gut hormones and glucose at all time points and triglycerides at time 0 minutes only (see table 2). Both groups will be given 200mL of water to consume with their snack food. A further 300mL of water will be provided over the 2-hour testing period. Participants will be required to drink all water provided. After 2 hours, a buffet meal will be provided, and participants will be advised to eat as much or as little as they like within 30 minutes. The buffet meal will be free of nuts and will provide a selection of core and noncore foods and beverages as defined by the Australian Dietary Guidelines for participants to select (47). The foods chosen from the buffet meal will be assessed for total energy consumed, macronutrient and micronutrient composition using Foodworks Nutritional Analysis Software version 9 (Xyris Software, Brisbane, QLD, Australia).

A protease inhibitor cocktail [protease inhibitor (Sigma P2714) and DPP-IV (Millipore DPP4-010)] will be added immediately to the blood sample intended for testing; glucagon-like peptide-1 (GLP1), ghrelin, leptin, pancreatic polypeptide (PP), glucose-dependent insulinotropic polypeptide (GIP), peptide tyrosine tyrosine (PYY), cholecystokinin (CCK), C-peptide, insulin and glucagon. The samples will be

analysed using a multiplex analysis system (LUMINEX MAGPIX, ThermoScientific). CCK will be assessed using ELISA (Ray Biotech) (%CV intra-assay <10%, inter-assay <15%) and insulin will be assessed at baseline only using Mercodia ELISA (%CV <5%) and insulin resistance will be calculated using the Homeostasis Model Assessment (HOMA2) Calculator v2.2.3 82. Glucose and triglycerides will be analysed via Konelab Auto Analyser (%CV <5%).

Subjective ratings of hunger, appetite and fullness will be measured by visual analogue scales (VAS) at time 0 (before the test snack) and every 30 minutes for 2 hours post the test snack, as well as post buffet meal. VAS scales will be presented on separate sheets and recorded to the nearest mm. VAS responses will be recorded on a 100 mm line and measured to the nearest mm as the distance from the left hand anchor, with “not/none at all /no desire” on the left and “extremely/great desire” on the right. Questions will include “How hungry do you feel?”, “How thirsty do you feel?”, “How satisfied do you feel?”, “How full do you feel?” and “How much do you think you can eat?”. The validity and reliability of this approach have previously been established (59, 60). Completed VAS score sheets will be removed, so participants are not able to see their previous scores. While participants will not be blinded to the food consumed, the researchers evaluating VAS data will remain blinded. Area under the curve (AUC) for responses to VAS scales (mm) will be plotted over time and calculated for each satiety/hunger measure using the trapezoidal estimation method (61).

Accelerometry

Physical activity will be measured using triaxial accelerometers (GENEActiv Original, Activinsights Ltd, UK), which will be worn on the non-dominant wrist. Participants will be asked to wear the monitor 24 hours/day for 14 consecutive days, removing it for showering/bathing or any other water-based activities. Devices will be configured through the manufacturer’s software (GENEActiv PC Software, Activinsights, UK) to record at 50 Hz for 14 days, starting at midnight of the first day of the monitoring period.

Participants will be provided with a paper-based record sheet to document; (1) the time they went to bed (“bedtime”), (2) the time they woke up (“get up time”) and (3) the time the device was removed (“non-wear”) and put back on again as well as the reason for removal (e.g., showing).

After the device is returned, the research team will download the raw acceleration data through the manufacturer's software. The Signal Vector Magnitude (SVM) of the acceleration, minus gravity, will be computed and summed over 60-second epochs:

$$SVM = \sum_{60s} \left| \sqrt{a_X^2 + a_Y^2 + a_Z^2} - g \right|$$ Where a_x, a_y, a_z are the three components of the acceleration signal and g the acceleration of gravity (9.81 m/s^2). The 60-second epoch data will then be imported into custom Matlab software for further processing. This software (*Cobra*, developed at the University of South Australia) provides a user-friendly graphical user interface for processing accelerometer data. Each 60-second epoch of waking wear time will be classified into one of four physical activity levels: sedentary, light, moderate or vigorous PA. Cutpoints for PA levels are defined according to Esliger et al. for adults (62) and adjusted proportionally to account for the 50 Hz sampling frequency (63). The resulting cutpoints between sedentary and light, light and moderate, and moderate and vigorous PA are 188, 403 and 1131 gravity units per minute (g.min), respectively. Device removals (non-wear) will be identified using the self-reported records and excluded from analysis. Where the reason given for removal is "sport", the removal period will be replaced with a period of MVPA.

Sleep will be identified using the self-reported records. Sleep times will be corrected by visual inspection when necessary, that is, in case sleep times were not reported or when obvious discrepancies were observed between reported sleep and accelerometer trace. Sleep quality will be assessed through total sleep time and sleep fragmentation (64). Each minute between "bed time" and "get up time" will be classified as sleep or wake using the algorithm developed by van Hees et al. to detect wake periods during the night (65). Total sleep time is the sum of all sleep minutes between "bedtime" and "get up time". Sleep fragmentation is the ratio of total sleep time over time in bed.

All sleep and physical activity variables will be averaged over monitoring days for each participant. Averages over weekdays (Monday-Friday) and weekend days (Saturday, Sunday) will also be computed to assess any potential differences in physical activity between the two. A day will be considered invalid and excluded from analysis if it included ≤10 hours wear during waking hours (66). A participant will be

considered invalid and excluded from analysis if they provide <4 valid days of accelerometry data (66, 67).

Resting Energy Expenditure

Resting energy expenditure (REE) will be measured using a ventilated hood system (TrueOne 2400 Metabolic System, ParvoMedics Inc, Sandy, UT, USA), which will be calibrated before each measurement using standardised gases. All testing will be conducted in the morning after a minimum 10 hour overnight fast. Testing will be performed in a thermo-neutral environment with participants lying supine in a comfortable position, head on a pillow, and a transparent ventilated hood placed over their head. During the measurement period, participants will be asked to remain as relaxed as possible without falling asleep and instructed not to talk or fidget. Oxygen consumption (VO_2) and carbon dioxide production (VCO_2) will be measured continuously for 30min. After discarding the first 10 min of data, REE will be calculated as the lowest consecutive 10 min average value, provided that the coefficient of variation within that 10 min interval is <5%. Resting energy expenditure will be calculated using the Weir equation [metabolic rate (kcal per day) = 1.44 (3.94 VO_2 + 1.11 VCO_2)] (68-70).

Doubly Labelled Water

Total daily energy expenditure during free-living conditions over 14 days will be quantified using the criterion doubly labelled water (DLW) technique at baseline, 3 months and 9 months in a subsample of participants (n=24 total, 12 per group). On each occasion, participants will be provided with a dose of isotope labelled water (10 atom% oxygen 18 (^{18}O) and 99.9 atom% deuterium (^{2}H)) with the dose based on body mass (1.35 g of DLW × body mass in kg). Participants will be asked to collect urine specimens daily over a 2-week period. Samples will be analysed by isotope ratio mass spectrometry (IRMS). Total daily energy expenditure (kJ) over the 2-week period will be divided by 14 to estimate mean total daily energy expenditure (71, 72).

Blood Pressure

At baseline, 3 months and 9 months, seated blood pressure will be recorded in a controlled environment using an automated sphygmomanometer and appropriately sized cuffs after a 5-minute quiet rest according to JNC 7 guidelines (73). The same

arm will be used for all assessment visits with the appropriately sized cuff. Four consecutive readings will be recorded at ~2 min intervals with the mean of the last three measurements used for analysis.

Flash Glucose Monitoring

Flash glucose monitoring will be used to assess dynamic changes in glucose over a 2 week period at baseline and at the end of both the weight loss and weight maintenance periods (FreeStyle Libre Flash Glucose Monitoring System) (74). Participants will wear a sensor on the back of their upper arm for up to 14 days and have a reader to scan the sensor every 6-8 hours. This system measures interstitial glucose concentrations and continuously stores measurement values every 15 minutes, which will provide information about postprandial glucose responses as well as changes in glucose regulation during weight loss. Average interstitial glucose and AUC (75) will be calculated and evaluated at baseline, 3 months and 9 months.

Dietary analysis

Participants will be asked to complete a 4-day weighed food record in the week prior to baseline appointments, in the last week of weight loss and the last week of weight maintenance phases. Participants will be asked to record all foods and drinks consumed during this time and to record weights or estimate volumes using standard measures where possible and provide as much detail as possible about branded products. Data will be collected on non-consecutive days and entered into Foodworks Nutritional Analysis Software version 8 (Xyris Software, Brisbane, QLD, Australia) for analysis of macronutrient and micronutrient intake as well as total energy intake.

Study Food Liking and Palpability Scores

A liking score for almonds and alternative snack foods will be assessed using a Labelled Affect Magnitude Scale (LAMS) (76)and a food action rating scale (FACT) (77) which will rate foods for overall liking, liking of textures and liking of flavours. These tests will occur at screening and at the end of weight loss and weight maintenance phase to determine any change following long-term consumption of the test foods.

Eating Behaviour, Mood and Personality

Eating behaviour, mood and personality will be assessed at baseline and at the end of the weight loss and weight maintenance phases. Change over time will be assessed using a series of validated questionnaires. Eating behaviour via; Power of Food (78), Food Craving Scale (79), Eating Attitude Test (80), Fickiness Questionnaire (81), EAT-26 (80), Control of Eating Questionnaire (82). Mood and personality via; Perceived Stress Scale (83), Zung Self-Rating Scale (84), Eysenck Personality Questionnaire (85), and Brief Sensation Seeking Scale (86).

Quality of Life, Functional Mobility and Pain

The Timed Up and Go (TUG) test is a test of functional mobility. This test requires participants to be timed while getting up, walking 3 meters, turning, returning to the chair, and sitting down again (87). Previous studies in adults have reported the CV error was 6% for the TUG test (88). The short-form 36 (SF36) questionnaire will be used for assessing overall quality of life, and pain will be assessed with the SF36 bodily pain subscale as well as a VAS scale to rate the intensity of pain at each major chronic and/or acute pain site. The nature of pain (at each site) will be rated using a short-form of the McGill pain questionnaire (89). All pain measures have been shown to be reliable and valid in adults (90), with the psychometrics of the SF-36 specific to the Australian population (91, 92).

Faecal Microbiota

Stool samples will be collected at baseline and following the weight loss and weight maintenance phases using OMNIgene.GUT DNA Stabilization Kits (DNA Genotek). DNA extraction will be performed using MoBio Powerlyzer Powersoil DNA Isolation Kit (MoBio Laboratories, Carlsbad, California) as described previously (93). DNA concentration will be quantified fluorometrically with a Qubit dsDNA HS Assay kit (Life Technologies). Faecal microbial composition will be assessed by 16S rRNA amplicon sequencing (94). This analysis will include determination of Firmicutes:Bacteroidetes ratios and quantification of *Akkermansia muciniphila* abundance. A subsample of high responders (n=10) and lowest responders (n=10) (based on weight regain) will also undergo more detailed analysis of samples from pre-baseline, end of weight loss and end of weight maintenance. Samples will be

assessed for identification of responder/non-responder phylogenetic and functional traits using shotgun metagenomic sequencing (95).

Liver Health

The amount of fat stored within the liver will be assessed using ultrasound. Participants will have their livers imaged using a clinical ultrasound scanner (Philips iU22 Ultrasound imaging system, Bothell, WA, USA) equipped with a 5-1 MHz high-resolution curved array transducer (Model c5-1, Philips). The amount of fat stored within the liver will be assessed using three different methods. Firstly, the size of the liver will be determined using the technique by Childs et al (96). Three linear measurements of the liver will be taken and then volume calculated by using the following equation: liver volume (cm^3) = 343.71 + [0.84 × ABC], where ABC is the product of the three linear measurements (96). Secondly, a visual assessment of fat accumulation will be evaluated according to the technique by Ballestri et al, adapted to liver images (97). A fatty liver indicator score ranging from 0 to 9 will be calculated using three indicators: (1) presence or absence of liver-kidney contrast; (2) assessment of beam penetration and (3) level of vessel wall blurring. Each measure will be graded as normal (score of 0), mild (score of 1), moderate (score of 2), or severe (score of 3) (97). Finally, quantification of liver fibrosis, a consequence of fatty liver, will be performed using shear wave elastography. The ultrasound machine emits a low-frequency pulse and software inbuilt into the ultrasound machine indicates the density of the liver tissue. The more fibrotic the tissue, the denser the tissue and the higher the reading (98).

Data Management

All participants will be given a unique code, which will be used to identify their electronic and paper-based data and biological samples. A password-protected computer database, accessible by the researchers only, will store participant identifiers (e.g., name, email address, phone number) and their associated code. Paper-based data will be stored securely at the University of South Australia for 15 years, after which it may be destroyed. Biological samples will be stored in a swipe card secured −80°C freezer, with an alarm system that alerts a staff member if the

temperature rises above a predetermined temperature. Samples will be stored for up to 15 years from collection date and disposed of accordingly after that time.

Protocol deviations

Protocol deviations will be communicated via an update of the Australian and New Zealand Clinical Trial Registry and also through a letter to the editor of this journal.

Adverse Events

Adverse events will be reported to the University of South Australia Human Research Ethics Committee.

Statistical analysis plan

Statistical analysis will be performed using SPSS for Windows 24.0 (SPSS, Chicago, IL, USA). Data will be tested for normality, and where possible non-normally distributed data will be log transformed prior to analysis. Characteristics between the AED and NFD groups at baseline will be compared using independent-sample t-test, Kruskal–Wallis tests or chi-squared tests (sex). The effects of diet treatment on the dependent measures over time will be analysed using random effects mixed models, with treatment (AED or NFD) and time (baseline, end of weight loss, and end of weight maintenance) being the between and within factors respectively in the analysis. Both intention-to-treat (ITT) and per-protocol analyses (for those who achieve a minimum of 80% compliance with test food consumption) will be completed. Where main effects are identified, Bonferroni post hoc tests will be performed to identify significant differences between means (P set at <0·05). Whilst the ITT analysis will be the main analysis, the per-protocol analysis will allow us to decipher that the effects are due to participants being compliant with consuming test foods. We will also run sub-analyses for both the ITT and per-protocol analyses which will stratify participants according to weight loss during the weight loss phase to determine whether there is an interaction between dietary treatment and weight loss in terms of effects on the various outcomes. This will allow for determination of whether almonds, compared with control, provide greater improvements in outcomes for any given level of weight loss.

Data Access

There are no contractual agreements that require the data from this trial to be shared.

Dissemination

Participants will receive all test foods and a copy of their individual results (with the exception of liver scans, faecal sample testing, resting energy expenditure, doubly labelled water and some blood analysis results) as well as a summary of the study findings. Participants who complete all aspects of the study will receive an honorarium of $400 to compensate for their time and travel expenses. The subsample of participants who also participate in doubly labelled water assessments will be provided with an additional $150 honorarium. Findings of the study will be disseminated at scientific conferences and published papers. Further, where appropriate outcomes will be made available to the public via media releases.

DISCUSSION

Nut consumption in many countries is low, and it has been suggested that people may avoid eating nuts as they may perceive that the high-fat content will lead to weight gain (12, 13, 15, 16). However, various mechanisms have been hypothesised regarding the association between nut consumption and weight loss success. Nuts are rich in protein, fibre and monounsaturated fat, macronutrients known to have a positive effect on appetite control (25). The high unsaturated fat content and protein in nuts may lead to an increase in resting energy expenditure and diet-induced thermogenesis (31). Additionally, the structure of the lipid-storing granules and high fibre content of nuts, as well as incomplete mastication may cause reduced fat absorption resulting in a loss of available energy (31). Despite this, clinical trials evaluating the effects of nut consumption compared to a nut-free diet on adiposity have reported mixed findings, with some studies reporting greater weight loss (17, 18) or improved body composition (19, 20), and others reporting no weight change (1, 5, 9), suggesting further investigation is required.

Strengths

There are a number of novel features in this study design. To our knowledge, this will be the first trial to assess whether the inclusion of 15% of energy from almonds in an otherwise nut-free diet will improve weight loss and limit weight regain. As such, this work has the potential to expand the current understanding of how regular consumption of almonds can aid weight loss and weight maintenance, while also providing beneficial cardiometabolic, liver and gut health effects. Furthermore, the metagenomic analysis that will be performed will be a substantial advance on our current understanding of the impact of almonds on the gut microbiome (previously limited to amplicon sequencing approaches) (99).

Limitations

One of the main challenges with any dietary intervention study is recruiting participants and keeping them motivated. We have previously successfully recruited large numbers of participants for similar intervention trials and plan to support participants with regular appointments with the dietitian, as well as the provision of test foods and simple food checklists to assist with compliance. A potential limitation of this study is that it will only be feasible to follow participants for 6 months after initial weight loss, although data from our recent pilot study suggests that this will be sufficient to observe differential changes in weight regain due to the rapidity of weight regain once intensive dietary support is removed (Wilson AL et al. Nudging Weight Loss Maintenance in Adults with Type-2 Diabetes: A Pilot Intervention).

This 9-month randomised controlled trial will add to the evidence base strategies to facilitate weight loss and prevent regain, potentially leading to recommendations to frequently substitute energy-dense snacks that lack nutritional value with nuts, which, in turn, could lead to improved weight loss outcomes and facilitate beneficial dietary habits (13, 100).

Author contributions: A.M.C, J.D.B, A.M.H, S.Y.T, G.B.R : were co-applicants on the grant application and as such were involved with the original design. A.C: was the lead applicant and is the principal investigator for the study. A.M.C, J.D.B, A.M.H, S.C, C.Y: are involved with study coordination and responsible for the day to day running of the trial, recruitment and sample collection. All authors: contributed to method development and the writing and development of the protocol paper and all authors will have responsibility for analysis, statistical interpretation of outcomes and preparation of manuscripts for publication post-study completion.

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Conflicts of interest: A.M.C, has consulted for Nuts for Life.

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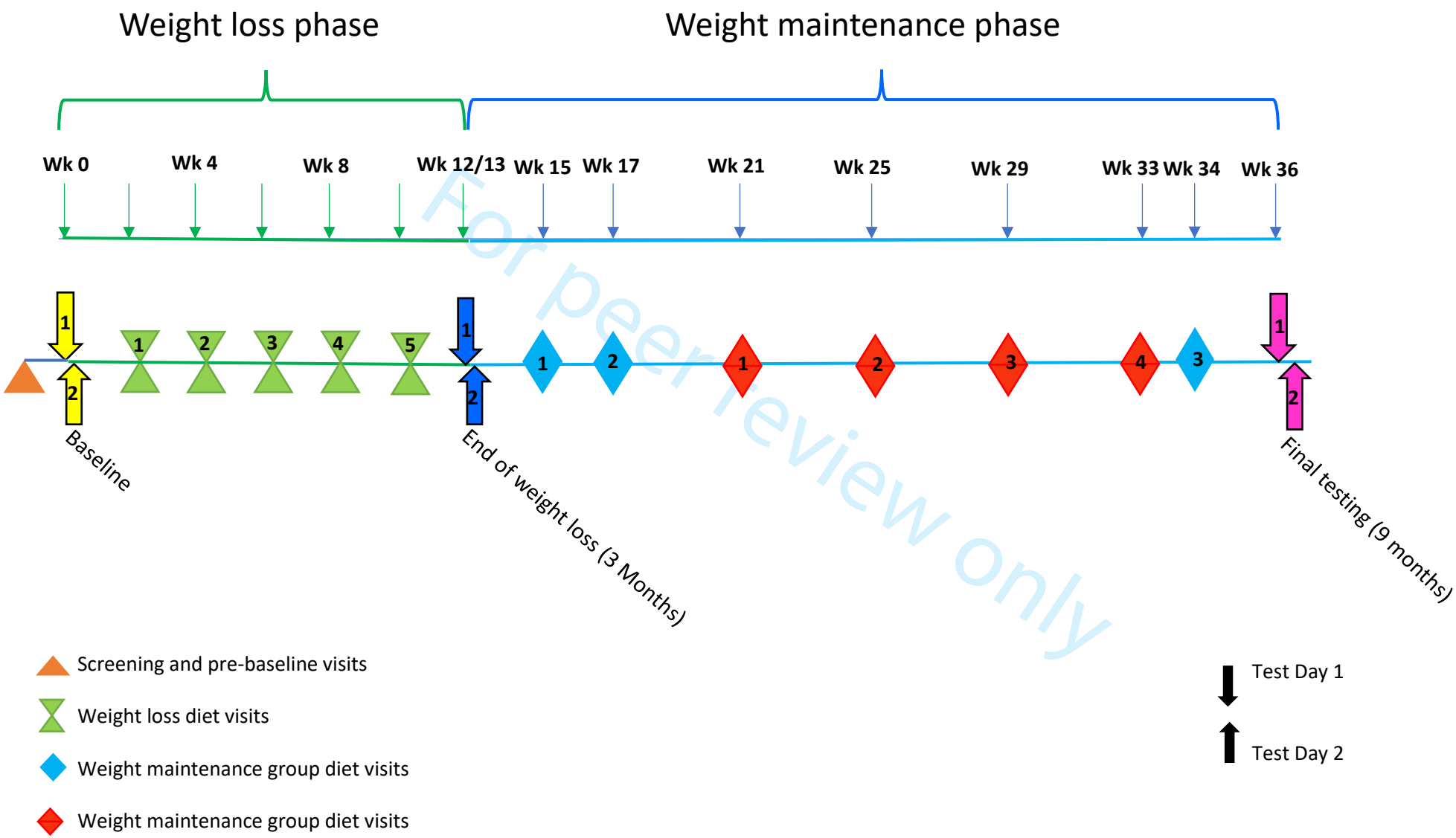
Figure 1: Study Timeline



SPIRIT
STANDARD PROTOCOL ITEMS: RECOMMENDATIONS FOR INTERVENTIONAL TRIALS

SPIRIT 2013 Checklist: Recommended items to address in a clinical trial protocol and related documents*

Section/item	Item No	Description	Addressed on page number
Administrative information			
Title	1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym	1
Trial registration	2a	Trial identifier and registry name. If not yet registered, name of intended registry	1
	2b	All items from the World Health Organization Trial Registration Data Set	N/A
Protocol version	3	Date and version identifier	1
Funding	4	Sources and types of financial, material, and other support	28
Roles and responsibilities	5a	Names, affiliations, and roles of protocol contributors	1
	5b	Name and contact information for the trial sponsor	1
	5c	Role of study sponsor and funders, if any, in study design; collection, management, analysis, and interpretation of data; writing of the report; and the decision to submit the report for publication, including whether they will have ultimate authority over any of these activities	28
	5d	Composition, roles, and responsibilities of the coordinating centre, steering committee, endpoint adjudication committee, data management team, and other individuals or groups overseeing the trial, if applicable (see Item 21a for data monitoring committee)	28

Introduction			
Background and rationale	6a	Description of research question and justification for undertaking the trial, including summary of relevant studies (published and unpublished) examining benefits and harms for each intervention	3-5
	6b	Explanation for choice of comparators	12
Objectives	7	Specific objectives or hypotheses	4-5
Trial design	8	Description of trial design including type of trial (eg, parallel group, crossover, factorial, single group), allocation ratio, and framework (eg, superiority, equivalence, noninferiority, exploratory)	5-7
Methods: Participants, interventions, and outcomes			
Study setting	9	Description of study settings (eg, community clinic, academic hospital) and list of countries where data will be collected. Reference to where list of study sites can be obtained	5
Eligibility criteria	10	Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and individuals who will perform the interventions (eg, surgeons, psychotherapists)	6
Interventions	11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered	12-13
	11b	Criteria for discontinuing or modifying allocated interventions for a given trial participant (eg, drug dose change in response to harms, participant request, or improving/worsening disease)	N/A
	11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return, laboratory tests)	12
	11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial	N/A
Outcomes	12	Primary, secondary, and other outcomes, including the specific measurement variable (eg, systolic blood pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg, median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen efficacy and harm outcomes is strongly recommended	13-24
Participant timeline	13	Time schedule of enrolment, interventions (including any run-ins and washouts), assessments, and visits for participants. A schematic diagram is highly recommended (see Figure)	9-11

Sample size	14	Estimated number of participants needed to achieve study objectives and how it was determined, including clinical and statistical assumptions supporting any sample size calculations	7
Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size	6-7
Methods: Assignment of interventions (for controlled trials)			
Allocation:			
Sequence generation	16a	Method of generating the allocation sequence (eg, computer-generated random numbers), and list of any factors for stratification. To reduce predictability of a random sequence, details of any planned restriction (eg, blocking) should be provided in a separate document that is unavailable to those who enrol participants or assign interventions	7
Allocation concealment mechanism	16b	Mechanism of implementing the allocation sequence (eg, central telephone; sequentially numbered, opaque, sealed envelopes), describing any steps to conceal the sequence until interventions are assigned	7
Implementation	16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions	7
Blinding (masking)	17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how	7
	17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial	7
Methods: Data collection, management, and analysis			
Data collection methods	18a	Plans for assessment and collection of outcome, baseline, and other trial data, including any related processes to promote data quality (eg, duplicate measurements, training of assessors) and a description of study instruments (eg, questionnaires, laboratory tests) along with their reliability and validity, if known. Reference to where data collection forms can be found, if not in the protocol	9-11, 13-24
	18b	Plans to promote participant retention and complete follow-up, including list of any outcome data to be collected for participants who discontinue or deviate from intervention protocols	6

Data management	19	Plans for data entry, coding, security, and storage, including any related processes to promote data quality (eg, double data entry; range checks for data values). Reference to where details of data management procedures can be found, if not in the protocol	24-25
Statistical methods	20a	Statistical methods for analysing primary and secondary outcomes. Reference to where other details of the statistical analysis plan can be found, if not in the protocol	25
	20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	25
	20c	Definition of analysis population relating to protocol non-adherence (eg, as randomised analysis), and any statistical methods to handle missing data (eg, multiple imputation)	25
Methods: Monitoring			
Data monitoring	21a	Composition of data monitoring committee (DMC); summary of its role and reporting structure; statement of whether it is independent from the sponsor and competing interests; and reference to where further details about its charter can be found, if not in the protocol. Alternatively, an explanation of why a DMC is not needed	N/A
	21b	Description of any interim analyses and stopping guidelines, including who will have access to these interim results and make the final decision to terminate the trial	N/A
Harms	22	Plans for collecting, assessing, reporting, and managing solicited and spontaneously reported adverse events and other unintended effects of trial interventions or trial conduct	25
Auditing	23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor	N/A
Ethics and dissemination			
Research ethics approval	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval	2
Protocol amendments	25	Plans for communicating important protocol modifications (eg, changes to eligibility criteria, outcomes, analyses) to relevant parties (eg, investigators, REC/IRBs, trial participants, trial registries, journals, regulators)	2 & 25

Consent or assent	26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)	7
	26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	N/A
Confidentiality	27	How personal information about potential and enrolled participants will be collected, shared, and maintained in order to protect confidentiality before, during, and after the trial	24
Declaration of interests	28	Financial and other competing interests for principal investigators for the overall trial and each study site	28
Access to data	29	Statement of who will have access to the final trial dataset, and disclosure of contractual agreements that limit such access for investigators	24
Ancillary and post-trial care	30	Provisions, if any, for ancillary and post-trial care, and for compensation to those who suffer harm from trial participation	N/A
Dissemination policy	31a	Plans for investigators and sponsor to communicate trial results to participants, healthcare professionals, the public, and other relevant groups (eg, via publication, reporting in results databases, or other data sharing arrangements), including any publication restrictions	26
	31b	Authorship eligibility guidelines and any intended use of professional writers	28
	31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	26
Appendices			
Informed consent materials	32	Model consent form and other related documentation given to participants and authorised surrogates	Yes, sent to editor
Biological specimens	33	Plans for collection, laboratory evaluation, and storage of biological specimens for genetic or molecular analysis in the current trial and for future use in ancillary studies, if applicable	24

*It is strongly recommended that this checklist be read in conjunction with the SPIRIT 2013 Explanation & Elaboration for important clarification on the items. Amendments to the protocol should be tracked and dated. The SPIRIT checklist is copyrighted by the SPIRIT Group under the Creative Commons "Attribution-NonCommercial-NoDerivs 3.0 Unported" license.

BMJ Open

Study protocol for a 9-month randomised controlled trial assessing the effects of almonds vs. carbohydrate-rich snack foods on weight loss and weight maintenance

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Study protocol for a 9-month randomised controlled trial assessing the effects of almonds vs. carbohydrate-rich snack foods on weight loss and weight maintenance.

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ABSTRACT

Introduction Epidemiological studies indicate an inverse association between nut consumption and body mass index (BMI). However, clinical trials evaluating the effects of nut consumption compared to a nut-free diet on adiposity have reported mixed findings with some studies reporting greater weight loss and others reporting no weight change. This paper describes the rationale and detailed protocol for a randomised controlled trial assessing whether the inclusion of almonds or carbohydrate-rich snacks in an otherwise nut-free energy-restricted diet will promote weight loss during 3 months of energy restriction and limit weight regain during 6 months of weight maintenance.

Methods and analysis One hundred and thirty-four adults aged 25-65 years with a BMI of 27.5-34.9kg/m^2 will be recruited and randomly allocated to either the almond-enriched diet (AED) [15% energy from almonds] or a nut-free control diet (NFD) [15% energy from carbohydrate-rich snack foods]. Study snack foods will be provided. Weight loss will be achieved through a 30% energy restriction over 3 months, and weight maintenance will be encouraged for 6 months by increasing overall energy intake by ~120-180 kcal/day as required. Food will be self-selected, based on recommendations from the study dietitian. Body composition, resting energy expenditure, total daily energy expenditure (via doubly labelled water), physical activity, appetite regulation, cardiometabolic health, gut microbiome, liver health, inflammatory factors, eating behaviours, mood and personality, functional mobility and pain, quality of life and sleep patterns will be measured throughout the 9-month trial. The effects of intervention on the outcome measures over time will be analysed using random effects mixed models, with treatment (AED or NFD) and time (baseline, 3 months, and 9 months) being the between and within factors respectively in the analysis.

Ethics and dissemination Ethics approval was obtained from the University of South Australia Human Research Ethics Committee (201436). Results from this trial will be disseminated through publication in peer-reviewed journals, national and international presentations.

Trial registration number ACTRN12618001861246; Pre-results.

Strengths and limitations of the study:

- To our knowledge, this will be the first randomised controlled trial to assess whether the inclusion of almonds vs. carbohydrate-rich snack foods in an otherwise nut-free diet will improve weight loss and limit weight regain.
- A wide range of outcomes will be assessed including but not limited to; body composition, resting and total daily energy expenditure, appetite regulation, cardiometabolic health, liver health, inflammatory markers and effects on the gut microbiome.
- Both objective and subjective appetite regulation will be evaluated, adding to our current limited knowledge of the effects of almonds on appetite control.
- The metagenomic analysis that will be performed will be a substantial advance on our current understanding of the impact of almonds on the gut microbiome (previously limited to amplicon sequencing approaches).
- A potential limitation of this study is that it will only be feasible to follow participants for 6 months after initial weight loss.

INTRODUCTION

Epidemiological studies report associations between increased frequency of nut consumption and lower body weight (1-4). This is supported by clinical data which suggests that regular nut intake has a beneficial impact on adiposity, insulin resistance and related metabolic abnormalities (5-11). Despite this, many people still avoid eating nuts due to the perception that they lead to weight gain based on their high energy and fat content. Nut consumption in many countries including Australia and America is low, (6 g/day and 3.3 g/day per capita, respectively) (12-14) with the prevalence of adult nut consumers being ~ 16-20% in Australia (15) and America (16). Such low consumption suggests there is scope to increase consumption in both countries.

Data from the National Health and Nutrition Examination Survey indicated that nut consumption was associated with a lower body mass index (BMI) (27.7 ± 0.2 kg/m^2 vs 28.1 ± 0.1 kg/m^2, $p < 0.05$) and waist circumference (95.6 ± 0.4 cm vs 96.4 ± 0.3 cm, $p < 0.05$), and tree nut consumers had lower body weight than non-consumers (78.8 ± 0.7 kg vs 80.7 ± 0.3 kg, $p < 0.05$) (16). When considering almonds specifically, randomised controlled trials have reported greater weight loss (17, 18) or improved body composition (reduced total fat and truncal fat) (19, 20) on hypocaloric diets with the inclusion of almonds compared to a nut-free diet. However, recent meta-analyses of clinical trials evaluating the effects of nut consumption on adiposity have reported no difference in body weight, BMI or waist circumference when comparing diets including nuts against control diets (5, 9). Nevertheless, changes in body fat distribution and reductions in fat stored in the liver can improve metabolic outcomes independent of weight changes. Inclusion of nuts may help prevent and manage non-alcoholic fatty liver disease (21, 22) and almond consumption has been associated with reductions in circulating liver enzyme concentrations (23). Additionally, data from large cohort studies suggest that frequent nut consumption may lower the risk of weight gain (4), with consumption on 5 or more days per week showing the greatest effect (24, 25). Prospective analysis of cohorts of healthy adults show that the average weight gain over 4 years was 3.3 lb (~1.5 kg). This weight gain was inversely associated with nut consumption (26).

Weight regain following initial weight loss is common and contributes, in part, to the obesity epidemic (27). Randomised controlled trials assessing weight loss maintenance report a 30-35% weight regain in the first year following a weight loss intervention with a 76% weight regain at 4 years post-treatment (28). Weight regain

suggests there is a need for nutritional strategies that prevent weight regain. Therefore assessing the effects of nutritional strategies, such as the inclusion of nuts for the prevention of weight regain, is paramount. Diets high in protein appear to limit weight regain (29) with higher intakes of non-cereal plant proteins, such as in nuts, associated with a protective effect (30). Nuts are rich in protein, fibre and monounsaturated fat, which have been suggested to contribute to their positive effect on appetite regulation (31, 32) and almonds, specifically, have been shown to have positive effects on subjective ratings of appetite (33, 34). However, the majority of assessments of appetite regulation with nuts have been conducted acutely and subjectively, and few have assessed appetite hormones (35). Reduced food cravings have been associated with long-term weight loss success (36); thus, assessing both of these outcomes following regular almond consumption is important.

Previous studies have reported significant differences in gut microbiota between lean and obese individuals (37) and modulation at the phylum and genus levels following weight loss (38). Changes in gut microbiota have also been observed following consumption of diets containing almonds (39) and almond skins (40), although not consistently in all populations (41). Significant increases in the relative abundance of bacterial taxa comprising of Ruminiclostridium, and members of the Ruminococcaceae and Lachnospiraceae families, under trial conditions are implicated in the degradation of complex dietary plant-derived polysaccharides, and the production of beneficial short-chain fatty acids (42). It has been suggested that bacterial products may impact on short-term intestinal satiety pathways and long-term appetite control, acting directly on hypothalamic neurons (43). However, it is unclear whether the changes in gut microbiota associated with almond consumption may help limit weight regain and the relationship with other biomarkers of cardiometabolic risk.

OBJECTIVES

Primary objective

The primary aim of this project is to evaluate whether inclusion of 15% of energy from almonds [almond-enriched diet (AED)] compared to carbohydrate-rich snacks in an otherwise nut-free energy-restricted diet [nut-free control diet (NFD)], will improve weight loss during 3 months of dietary energy restriction and limit weight regain during 6 months of weight maintenance. We hypothesize that the AED will lead to greater

weight loss during the energy restriction phase of 3 months and limit weight regain during the weight maintenance period of 6 months compared to the NFD.

Secondary objectives

The secondary aim is to evaluate whether an AED compared to a NFD improves body composition and body fat distribution (reduced waist circumference and abdominal fat depots), impacts resting and total daily energy expenditure and improves subjective and objective measures of satiety.

Tertiary objectives

The tertiary aim is to evaluate whether an AED compared to a NFD reduces fat accumulation in the liver and improves liver enzyme profiles, results in beneficial changes in the composition of the gut microbiome, improves inflammatory biomarkers and cardiometabolic health outcomes including blood lipid profiles, glucose and insulin, and improves self-reported eating behaviours, mood, personality, pain, functional mobility, quality of life, sleep and physical activity patterns.

METHODS AND ANALYSIS

Study design

The study is designed as a 9-month randomised controlled parallel-arm dietary intervention. The study will be conducted in the research facilities of the Alliance for Research in Exercise, Nutrition and Activity (ARENA) at the University of South Australia, Adelaide. The SPIRIT guidelines were used in the development of this protocol (44).

Patient and public involvement

Development of this research protocol was done without patient involvement. The final study results will be disseminated to all participants.

Participants

Eligibility criteria

Participants will be male and female volunteers, aged 25-65 years, with a BMI of 27.5-34.9 kg/m^2. Age range ensures physical maturity has been achieved and limits the possibility of chronic health conditions that would exclude the volunteer from participation. BMI range ensures sufficient weight available to lose and reduces the risk of chronic health conditions. Participants will be non-smokers (minimum 6 months) and weight stable (within 5 kg) for 3 months prior to enrolment. Detailed inclusion and exclusion and withdrawal criteria listed in table 1.

Table 1. Eligibility Criteria

Inclusion Criteria
Males and females aged 25-65 years
Body mass index (BMI) 27.5-34.9 kg/m^2
Weight stable (within 5kg) in the past 3 months
Non-smoker (minimum 6 months)
Exclusion Criteria
Cardiovascular Disease
Type 1 or Type 2 Diabetes
Thyroid Disorders
Kidney or Liver disease
Gastrointestinal disorders requiring medical nutrition therapy (e.g. Crohn's disease, irritable bowel, coeliac disease)
Are pregnant or breastfeeding
Allergies to nuts, gluten or other components of the test foods
Unable to chew hard foods such as nuts
Consumed more than 30g of nuts per week in the month prior to beginning the trial
Alcohol (>14 standard drinks/week) (1 standard drink = 100 ml wine, 285 ml beer, 30 ml spirit) or drug dependency
Have changed medications or supplements in the last 3 months
Take vitamin, mineral, herbal supplementation or medications that may have an impact on study outcomes
Unwilling to stop dietary supplements that influence weight
Suffer claustrophobia or a fear of enclosed spaces
Show unwillingness to be randomised to either experimental groups (based on liking and palatability questionnaires)
Withdrawal Criteria
Adverse reaction to test foods
The need to take a medication or treatment, which in the opinion of the investigator, may interfere with study measurements
Consistent non-compliance (<80% compliance) with consuming study foods.
Failure to satisfy the investigator regarding suitability to participate for any other reason

Recruitment/screening

Participants will be recruited from the public through radio, TV, printed media, internet-based advertisements and flyer distribution. Procedures will occur in accordance with ethical standards, including obtaining written informed consent.

Interested participants will be sent the participant information and a diet and lifestyle questionnaire (DLQ) to determine eligibility. Participants who appear eligible from DLQ responses will be assigned a screening number and will undergo an initial screening interview over the telephone to review medical history, concomitant medication and supplementation. Prospective participants will attend the clinical research facility approximately 3 weeks before baseline where eligibility will be confirmed, and the likelihood to consume test foods will be established via liking and palatability questionnaires (see Table 2). Participants who meet the required criteria and are deemed eligible, will be asked to provide written informed consent in the presence of the investigator and will proceed with a pre-baseline clinic appointment, 2 weeks prior to baseline.

Randomisation, allocation concealment and sequence generation

Data collected at the screening visit will be used to assign participants to groups based on age, sex, and BMI in the process of randomisation by minimization (45). Minimisation will ensure baseline characteristics are balanced between the treatment groups and has been proposed to be the most suitable randomisation method for small clinical trials, such as the proposed study, to reduce bias (45, 46). A staff member independent of the study outcome assessments and statistical analysis will perform the treatment allocation and maintain the randomisation list in a secure location with access limited to authorised personnel. As the participants are consuming whole foods, which are easily identified, the participants and staff involved in diet management cannot be blinded. Staff conducting clinical assessments at baseline, 3 months and 9 months will remain blinded to treatment group allocation. Participants will be asked not to disclose the foods they are consuming to the researchers. Researchers conducting assessments and analysing data will remain blinded until the completion of statistical analysis.

Sample size calculation

The study is powered on the primary outcomes of weight loss and weight regain. One hundred participants will provide 80% power to detect a 2.4 kg difference in weight loss (18) and a 1.7 kg difference in weight regain (based on variance in our pilot data) (Wilson AL et al. Nudging Weight Loss Maintenance in Adults with Type-2 Diabetes: A Pilot Intervention) between the two groups (α-level of 0.05). One hundred and thirty-four participants will be recruited (n=67 in each group) to allow for a 25% dropout.

Pre-intervention

Two weeks prior to baseline visits participants will be asked to attend a pre-baseline session. During this visit, a flash blood glucose monitoring sensor (FreeStyle Libre, Abbott, Australia) will be inserted for collection of blood glucose measures for 2 weeks, a wrist-worn accelerometer (GENEActiv, Activinsights Ltd, UK) will be provided for measuring physical activity and sleep patterns for 2 weeks, and a test kit will be provided for a one-off stool sample collection. Participants will be asked to keep a 4-day weighed food diary (non-consecutive days with 1 weekend day) and a 14-day sleep diary. Several questionnaires will also be administered to assess eating behaviour, mood, stress and personality (see Table 2).

Table 2. Outcome Measures at Each Time Point

	Screening	Pre-baseline	Baseline		Study Period				Close-out	
Time (Weeks) from Start of Dietary Intervention	**-3**	**-2**	**-1(D1)**	**0 (D2)**	**10**	**12 (D1)**	**13 (D2)**	**35**	**36 (D1)**	**37 (D2)**
Study Food Liking & Palatability Scores										
Labelled Affect Magnitude Scale	X								X	
Food Action Rating Scale	X								X	
Body Composition										
Height	X	X								
Weight (& Body Mass Index)	X	X	X	X	X	X	X	X	X	X
Total and Truncal Fat Mass (DXA)			X			X			X	
Total and Truncal Fat-Free Mass (DXA)			X			X			X	
Visceral Adipose Tissue (DXA)			X			X			X	
Waist Circumference			X			X			X	
Energy Expenditure										
Accelerometry		X			X			X		
Resting Energy Expenditure (Indirect Calorimetry)			X			X			X	
Total Daily Energy Expenditure (Doubly Labelled Water)[a]		X				X			X	
International Physical Activity Questionnaire			X			X			X	
Appetite Regulation and Eating Behaviour										
Energy Intake										
4-day Food Diary		X			X			X		
24-hour Diet Recalls[b]							X			X
Fasting and Postprandial Gut Hormones & Glucose										
Glucagon-like peptide-1				X			X			X
Ghrelin				X			X			X
Leptin				X			X			X
Pancreatic Polypeptide				X			X			X

	Screening	Pre-baseline	Baseline		Study Period				Close-out	
Time (Weeks) from Start of Dietary Intervention	**-3**	**-2**	**-1(D1)**	**0 (D2)**	**10**	**12 (D1)**	**13 (D2)**	**35**	**36 (D1)**	**37 (D2)**
Glucose-Dependent Insulinotropic Polypeptide (Gastric Inhibitory Polypeptide)				X			X			X
Peptide Tyrosine Tyrosine (Peptide YY)				X			X			X
C-Peptide				X			X			X
Cholecystokinin (CCK)				X			X			X
Glucagon				X			X			X
Glucose				X			X			X
Drive to Eat and Eating Behaviour										
Subjective Drive to Eat (Visual Analogue Scales) – Fasting and Postprandial				X			X			X
Energy Consumed at a Buffet Meal				X			X			X
Power of Food Survey			X			X			X	
Food Craving Scale			X			X			X	
Pickiness/Fickiness Questionnaire			X						X	
Eating Attitude Test (EAT-26)			X			X			X	
Control of Eating Questionnaire			X			X			X	
Psychology and Health										
General Health, Pain, Mobility, Mood and Personality										
Short-form 36 (SF36) Questionnaire				X			X			X
Profile of Mood States (POMS)				X			X			X
Perceived Stress Scale		X				X			X	
Zung Self-Rating Scale		X				X			X	
McGill Pain Scale and Chronic & Acute Pain Scales (Visual Analogue Scales)				X			X			X
Timed Up and Go (Functional Mobility)			X			X			X	
Eysenck Personality Questionnaire		X								
Brief Sensation Seeking Scale		X								
Gut Health										
Faecal microbiome composition		X			X			X		
Liver Health										

	Screening	Pre-baseline	Baseline		Study Period				Close-out	
Time (Weeks) from Start of Dietary Intervention	**-3**	**-2**	**-1(D1)**	**0 (D2)**	**10**	**12 (D1)**	**13 (D2)**	**35**	**36 (D1)**	**37 (D2)**
Liver Ultrasound				X			X			X
Alanine Aminotransferase (ALT)			X			X			X	
Aspartate Aminotransferase (AST)			X			X			X	
Alkaline Phosphatase (ALP)			X			X			X	
γ-Glutamyltransferase (GGT)			X			X			X	
Cardiometabolic Health										
Blood pressure	X	X	X			X			X	
Flash Glucose Monitoring		X			X			X		
Insulin				X			X			X
HOMA				X			X			X
Triglycerides			X	X		X	X		X	X
Cholesterols (TC, HDL-C, LDL-C, VLDL-C, IDL-C, and subclasses, Lipoprotein (a), Oxidised LDL, LDL-C particle size)			X			X			X	
Apolipoprotein B			X			X			X	
Apolipoprotein A1			X			X			X	
Inflammatory Markers										
F2-Isoprostane levels (plasma + urine)			X			X			X	
C-reactive protein			X			X			X	
Adiponectin			X			X			X	
Sleep Patterns										
Pittsburg Sleep Quality Index			X			X			X	
14-day sleep diary		X			X			X		
Biomarkers of Compliance										
αlpha-tocopherol			X			X			X	

[a] Sub-sample only; [b] 3 x 24-hour recalls were completed at random intervals between 0-13 weeks and 13-37 weeks; D1, day 1; D2, day 2; DXA, dual-energy X-ray absorptiometry; HDL-C, high density lipoprotein-cholesterol; IDL-C, intermediate density lipoprotein-cholesterol; LDL-C, low density lipoprotein-cholesterol; TC, total cholesterol; VLDL-C, very low density lipoprotein-cholesterol.

Study Intervention

The intervention will be a 9-month protocol, consisting of 3 months weight loss and 6 months weight maintenance. During the 9-month study period, participants in the AED will incorporate 15% of their energy as unsalted, whole, natural almonds with skins while participants in the NFD will include 15% of their energy consumed as carbohydrate-rich snack foods (oven-baked fruit cereal bar and rice crackers) as part of a 30% energy restricted weight loss diet. It is expected that the minimum quantity of almonds required to contribute 15% of energy will be 30 g, which is consistent with dietary guidelines (47). The control foods have been selected as they are commonly consumed snacks, and do not contain the beneficial micro and macro-nutrients available in almonds but can provide equal energy density (see Table 3). Participants will be provided with test foods to consume 6 days per week so that they have 1 day per week free from consuming test food. This has previously been found to support compliance (48). Checklists will be used to record daily consumption of study food and participants will be asked to return leftover food to calculate compliance scores. The threshold of compliance with test food consumption is >80%. All participants will be asked to avoid all other nuts and nut products during the entire study.

Table 3. Macronutrient Composition of Test Foods

Per 100g	Almonds	Weight Watchers Apple Crumble Bar	Rice Crackers
Energy (kJ)	2385.0	1270.0	1646.6
Protein (g) (%)	19.7 (14.0)	4.4 (5.9)	9.4 (9.8)
Total fat (g) (%)	50.5 (78.3)	1.0 (2.9)	5.6 (12.5)
Saturated fat (g)	3.8	0.3	1.4
Polyunsaturated fat (g)	12.8	0.3	1.2
Monounsaturated fat (g)	30.7	0.3	2.6
Carbohydrate (g) (%)	5.4 (3.6)	55.7 (72.4)	74.6 (76.8)
Sugars (g)	5.2	27.5	1.7
Starch (g)	0.2	28.2	72.8
Fibre (g) (%)	10.9 (3.7)	14.7 (9.3)	1.8 (0.9)

Energy requirements will be determined by using the Schofield Equation, based on age, sex, and baseline body weight, as well as self-reported physical activity captured via the International Physical Activity Questionnaire (IPAQ) to determine physical activity level (PAL) (49). Energy recommendation for weight loss will be 30% less than requirements to achieve 0.5-1 kg weight loss per week. Participants will be guided to consume a variety of foods within all five food groups to ensure they are still meeting nutrient reference guidelines consistent with Australian Dietary Guidelines (47). Participants will be provided with food group serve advice consistent with an energy restriction plan closest to their weight loss energy requirement of either 1200, 1500 or 1800 kcal/day (5000, 6300 or 7600 kJ/day). Weight loss plans will be adapted if energy requirements are substantially higher than 7600 kJ/day. We will lend participants a set of kitchen food scales to weigh food to ensure serve size accuracy. Three sample meal plans will be provided, as well as a recommended discretionary serve allowance of 2 per week, consistent with their energy requirement. Diet checklists will be used to assist with dietary compliance. During the weight maintenance phase, participants will be encouraged to stabilise their weight by increasing their overall energy intake by ~120-180 kcal/day (~500-750 kJ/day), with additional adjustments as required through dietetic consultation.

Participants will attend two baseline appointments (Day 1, -1 week and Day 2 – 0 weeks before intervention) and will be asked to refrain from alcohol for 24 hours and fast for a minimum of 10 hours prior to assessments. Details of tests, assessments and outcome measures completed on Days 1 and 2 are available in Table 2. Participants will meet with the study dietitian at the end of the Day 2 appointment to receive initial dietary counselling and instructions on test food consumption requirements. They will then meet individually with a dietitian every 2 weeks during the weight loss phase to have their weight monitored and test food compliance checked. During weight maintenance, participants will meet individually with the dietitian every 2 weeks for the first month and then monthly in small groups. Figure 1 outlines the study timeline. Adherence to energy-restricted diets will be assessed using 3 x 24-hour dietary recalls (via phone, at random times) during each phase, with weighed food diaries at baseline and at the end of the weight loss and weight maintenance phases. Participants will also be encouraged to meet national physical activity guidelines for Australian adults - 150 to 300 minutes (2 ½ to 5 hours) of moderate intensity physical activity or equivalent, per week (47). Accelerometer data, as well as self-reported activity via the IPAQ, will allow us to monitor whether 'unexpected' weight change might be explained by physical activity levels, and will assist in understanding the weight loss effects attributable to consuming almonds. Two weeks before the end of the weight loss and weight maintenance phases, participants will repeat testing that occurred at pre-baseline and baseline Day 1 and Day 2 (see Table 2).

Data Collection

The following section outlines the data and biochemical samples being collected during the test periods (see Table 2 for a summary).

Anthropometry

All anthropometric assessments will be conducted with participants barefoot and wearing light clothing. Height will be measured twice to the nearest 1 mm with the average value calculated and recorded using a stadiometer at baseline (SECA 216 Height Measuring Rod, SECA). Body weight will be recorded to the nearest 100 g following an overnight fast and will be measured twice on each occasion using calibrated electronic scales (SECA 703 wireless column scales, SECA) and the average value calculated. The same scales will be used throughout the intervention.

BMI will be calculated as weight/height squared (kg/m^2). Waist circumference will be measured, according to the protocol of the International Society for the Advancement of Kinanthropometry (50), using a metal measuring tape at the narrowest point of the abdomen or, if there is no obvious narrowing, at the midpoint between the lower costal (10th rib) border and the iliac crest. Two measurements will be taken unless they differ by more than 2% whereby a third measurement will be obtained. The mean of the measurements will be used for analysis. Body composition will be determined from a whole body dual-energy x-ray absorptiometry (DXA) scan, (Lunar ProdigyModel, General Electric, Madison WI USA). Participants will wear a light disposable gown, and all external metal objects will be removed. Total body fat mass (%, kg), total body lean mass (%, kg), regional fat mass (arms, legs, trunk and abdominal fat mass, android, gynoid (kg)) and visceral adipose tissue amount and volume (kg and cm^3), will be obtained using enCORE™ 2015 software (GE Healthcare enCORE version 13.31).

In addition to the measurements taken at baseline, at the end of weight loss and weight maintenance phases, weight will also be measured during dietetic counselling appointments to provide feedback to participants. We will also lend participants scales (Withings/Nokia WBS06, Nokia) with Bluetooth capacity to enable them to monitor their weight at least twice per week at home and for these data to be sent to research staff to assist with weight monitoring. Regular weight monitoring has been shown to enhance success in weight loss (51) and weight maintenance trials (52).

Biochemical Measures

At Day 1 baseline, 3-month and 9-month appointments, fasting (>10 hours) venous blood samples will be taken by a trained phlebotomist. Collected blood samples will be centrifuged (4° C, 4000 rpm, 10 mins) to separate plasma or serum and stored at −80°C for later analysis (see Table 4).

Table 4. Blood and Faecal Analysis

Parameter	Analysis Method	Sample Collected (Additives)
TC	Vertical Auto Profile (VAP II) (53)	Serum
HDL-C + subclass	Vertical Auto Profile (VAP II) (53)	Serum
LDL-C + subclass	Vertical Auto Profile (VAP II) (53)	Serum
IDL-C + subclass	Vertical Auto Profile (VAP II) (53)	Serum
VLDL-C + subclass	Vertical Auto Profile (VAP II) (53)	Serum
Lipoprotein(a)	Vertical Auto Profile (VAP II) (53)	Serum
Oxidised LDL-C	Solid phase 2-site ELISA	Serum
LDL particle number and size	Nuclear magnetic resonance (NMR) spectroscopy (54)	Serum
Triglyceride	Konelab Auto Analyser	Plasma
APOB	Vertical Auto Profile (VAP II) (53) and patented equations (20)	Serum
APOA1	Vertical Auto Profile (VAP II) (53) and patented equations (20)	Serum
hs-CRP	Konelab Auto Analyser	Serum
Adiponectin	ELISA	Serum
F2-Isoprostanes	Electron-capture negative-ion gas chromatography-mass spectrometry (55)	Plasma + Urine (Butylated Hydroxytoluene)
Alpha-tocopherol	High-performance liquid chromatography using the photo-diode array method (56)	Plasma
ALT	Abbott Alinity C	Serum
AST	Abbott Alinity C	Serum
ALP	Abbott Alinity C	Serum
GGT	Abbott Alinity C	Serum
Glucose	Konelab Auto Analyser	Plasma
Insulin	Mercodia ELISA	Plasma (Protease inhibitor and DPP-IV)
HOMA	Calculated using the Homeostasis Model Assessment Calculator v.2.3.3 (57)	-
Glucagon	Multiplex analysis system	Plasma (Protease inhibitor and DPP-IV)
GLP-1	Multiplex analysis system	Plasma (Protease inhibitor and DPP-IV)
Ghrelin	Multiplex analysis system	Plasma (Protease inhibitor and DPP-IV)
Leptin	Multiplex analysis system	Plasma (Protease inhibitor and DPP-IV)

Pancreatic Polypeptide	Multiplex analysis system	Plasma (Protease inhibitor and DPP-IV)
GIP	Multiplex analysis system	Plasma (Protease inhibitor and DPP-IV)
PYY	Multiplex analysis system	Plasma (Protease inhibitor and DPP-IV)
C-Peptide	Multiplex analysis system	Plasma (Protease inhibitor and DPP-IV)
CCK	ELISA (Ray Biotech)	Plasma (Protease inhibitor and DPP-IV)
Faecal Microbiota	MoBio Powerlyzer Powersoil DNA Isolation Kit	OMNIgene GUT DNA Stabilization Kit

ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; APOA1, apolipoprotein A1; APOB, apolipoprotein B; CCK, cholecystokinin; GGT, γ-Glutamyltransferase; GLP-1, glucagon-like peptide-1; GIP, glucose-dependent insulinotropic polypeptide (gastric inhibitory polypeptide); HDL-C, high density lipoprotein-cholesterol; hs-CRP, high-sensitivity c-reactive protein; IDL-C, intermediate density lipoprotein-cholesterol; LDL-C, low density lipoprotein-cholesterol; PYY, peptide tyrosine tyrosine (peptide YY); TC, total cholesterol; VLDL-C, very low density lipoprotein-cholesterol.

Lipids

Serum lipid, cholesterol lipoprotein subclasses, and apolipoproteins will be assayed using Vertical Auto Profile (VAP II) (Atherotech Diagnostics Lab, Birmingham, AL), which directly measures cholesterol in all lipoprotein classes (53). Serum lipoprotein particle number and size will be assessed by a proton magnetic resonance spectroscopy assay (NMR LipoProfile III; LipoScience, Raleigh, NC), which measures the particle concentrations of lipoprotein subclasses and average particle size of lipoproteins (54). This assay quantifies cholesterol concentrations of total lipoprotein, HDL, LDL, very-low-density lipoprotein (VLDL), lipoprotein(a) [Lp(a)], intermediate-density lipoprotein (IDL), and HDL, LDL, VLDL, and IDL subclasses. ApoB and ApoA1 will be calculated using results from the VAP test and patented equations (20). Serum oxidised LDL will be measured in duplicate by a solid phase 2-site ELISA (Mercodia, Uppsala, Sweden). Plasma triglyceride (%CV <5%) will be measured using a Konelab Auto Analyser.

Inflammatory Markers

Fasting plasma and a spot urine sample will be collected for analysis of F2-Isoprostanes as biomarkers of oxidative stress. Samples will be stabilised with butylated hydroxytoluene (BHT). Plasma and urine F2-Isoprostanes will be measured as total (free plus esterified) F2-Isoprostanes using electron-capture negative-ion gas chromatography–mass spectrometry as described previously (55). Serum will be collected for assessment of adiponectin by ELISA (Linco Research, St. Charles, Missouri) (58) and high-sensitivity C-reactive protein (hs-CRP) (%CV intra-assay 2.9%, inter-assay 1.9%) will be measured using a Konelab Auto Analyser.

Liver Enzymes

Fasting serum levels of alanine aminotransferase (ALT) (%CV intra-assay 0.5%, inter-assay 1.0%), aspartate aminotransferase (AST) (%CV intra-assay 0.6%, inter-assay 0.8%), alkaline phosphatase (ALP) (%CV intra-assay 0.3%, inter-assay 1.7%) and γ-glutamyltransferase (GGT) (%CV intra-assay 0.4%, inter-assay 1.1%) and will be measured using a local pathology service (23). To eliminate the effect of freeze-thawing of samples that may lower enzyme activity values, ALT, AST, ALP and GGT testing will be conducted on samples immediately transferred to the pathology laboratory.

Biomarkers of Compliance

Compliance with long-term almond consumption can be confirmed by measuring alpha-tocopherol levels (59, 60). Plasma alpha-tocopherol levels will be analysed using high-performance liquid chromatography with photo-diode array method according to Liu et al (56).

Appetite Regulation

At Day 2 baseline, 3-month and 9-month, objective and subjective measures of appetite and satiety will be assessed. A test snack will be consumed following an overnight fast (>10 hours). The test snack for the AED group will comprise 15% of daily energy intake from almonds, and the NFD group will have 15% of daily energy intake from a high carbohydrate snack (oven-baked fruit filled bar). Blood samples will be obtained at time 0 (before the test snack) and every 30 minutes for 2 hours post the test snack, with participants asked to consume the test snack within 10 minutes. Blood samples will be collected via a BD Nexiva™ cannula blood collection system by a trained phlebotomist. Collected blood samples will be centrifuged (4° C, 4000 rpm, 10 mins) to separate plasma or serum and stored at −80°C for later analysis of gut hormones and glucose at all time points and triglycerides at time 0 minutes only (see Table 4). Both groups will be given 200mL of water to consume with their snack food. A further 300mL of water will be provided over the 2-hour testing period. Participants will be required to drink all water provided. After 2 hours, a buffet meal will be provided, and participants will be advised to eat as much or as little as they like within 30 minutes. The buffet meal will be free of nuts and will provide a selection of core and noncore foods and beverages as defined by the Australian Dietary Guidelines for participants to select (47). The foods chosen from the buffet meal will be assessed for total energy consumed, macronutrient and micronutrient composition using Foodworks Nutritional Analysis Software version 9 (Xyris Software, Brisbane, QLD, Australia).

A protease inhibitor cocktail [protease inhibitor (Sigma P2714) and DPP-IV (Millipore DPP4-010)] will be added immediately to the blood sample intended for testing; glucagon-like peptide-1 (GLP1), ghrelin, leptin, pancreatic polypeptide (PP), glucose-dependent insulinotropic polypeptide (GIP), peptide tyrosine tyrosine (PYY), cholecystokinin (CCK), C-peptide, insulin and glucagon. The samples will be analysed

using a multiplex analysis system (LUMINEX MAGPIX, ThermoScientific). CCK will be assessed using ELISA (Ray Biotech) (%CV intra-assay <10%, inter-assay <15%) and insulin will be assessed at baseline only using Mercodia ELISA (%CV <5%) and insulin resistance will be calculated using the Homeostasis Model Assessment (HOMA2) Calculator v2.2.3 82. Glucose and triglycerides will be analysed via Konelab Auto Analyser (%CV <5%).

Subjective ratings of hunger, appetite and fullness will be measured by visual analogue scales (VAS) at time 0 (before the test snack) and every 30 minutes for 2 hours post the test snack, as well as post buffet meal. VAS scales will be presented on separate sheets and recorded to the nearest mm. VAS responses will be recorded on a 100 mm line and measured to the nearest mm as the distance from the left hand anchor, with “not/none at all /no desire” on the left and “extremely/great desire” on the right. Questions will include “How hungry do you feel?”, “How thirsty do you feel?”, “How satisfied do you feel?”, “How full do you feel?” and “How much do you think you can eat?”. The validity and reliability of this approach have previously been established (61, 62). Completed VAS score sheets will be removed, so participants are not able to see their previous scores. While participants will not be blinded to the food consumed, the researchers evaluating VAS data will remain blinded. Area under the curve (AUC) for responses to VAS scales (mm) will be plotted over time and calculated for each satiety/hunger measure using the trapezoidal estimation method (63).

Accelerometry

Physical activity will be measured using triaxial accelerometers (GENEActiv Original, Activinsights Ltd, UK), which will be worn on the non-dominant wrist. Participants will be asked to wear the monitor 24 hours/day for 14 consecutive days at baseline, 3 months and 9 months (see Table 2), removing it for showering/bathing or any other water-based activities. Devices will be configured through the manufacturer’s software (GENEActiv PC Software, Activinsights, UK) to record at 50 Hz for 14 days, starting at midnight of the first day of the monitoring period.

Participants will be provided with a paper-based record sheet to document; (1) the time they went to bed (“bedtime”), (2) the time they woke up (“get up time”) and (3) the time the device was removed (“non-wear”) and put back on again as well as the reason for removal (e.g., showering).

After the device is returned, the research team will download the raw acceleration data through the manufacturer's software. The Signal Vector Magnitude (SVM) of the acceleration, minus gravity, will be computed and summed over 60-second epochs:

$$SVM = \sum_{60s} \left| \sqrt{a_X^2 + a_Y^2 + a_Z^2} - g \right|$$ Where a_x, a_y, a_z are the three components of the acceleration signal and g the acceleration of gravity (9.81 m/s^2). The 60-second epoch data will then be imported into custom Matlab software for further processing. This software (*Cobra*, developed at the University of South Australia) provides a user-friendly graphical user interface for processing accelerometer data. Each 60-second epoch of waking wear time will be classified into one of four physical activity levels: sedentary, light, moderate or vigorous PA. Cutpoints for PA levels are defined according to Esliger et al. for adults (64) and adjusted proportionally to account for the 50 Hz sampling frequency (65). The resulting cutpoints between sedentary and light, light and moderate, and moderate and vigorous PA are 188, 403 and 1131 gravity units per minute (g.min), respectively. Device removals (non-wear) will be identified using the self-reported records and excluded from analysis. Where the reason given for removal is "sport", the removal period will be replaced with a period of moderate to vigorous physical activity.

Sleep will be identified using the self-reported records. Sleep times will be corrected by visual inspection when necessary, that is, in case sleep times were not reported or when obvious discrepancies were observed between reported sleep and accelerometer trace. Sleep quality will be assessed through total sleep time and sleep fragmentation (66). Each minute between "bed time" and "get up time" will be classified as sleep or wake using the algorithm developed by van Hees et al. to detect wake periods during the night (67). Total sleep time is the sum of all sleep minutes between "bedtime" and "get up time". Sleep fragmentation is the ratio of total sleep time over time in bed.

All sleep and physical activity variables will be averaged over monitoring days for each participant. Averages over weekdays (Monday-Friday) and weekend days (Saturday, Sunday) will also be computed to assess any potential differences in physical activity between the two. A day will be considered invalid and excluded from analysis if it included ≤10 hours wear during waking hours (68). A participant will be considered invalid and excluded from analysis if they provide <4 valid days of accelerometry data

over the 14 day testing period at baseline, 3 months or 9 months (see Table 2) (68, 69).

Resting Energy Expenditure

Resting energy expenditure (REE) will be measured using a ventilated hood system (TrueOne 2400 Metabolic System, ParvoMedics Inc, Sandy, UT, USA), which will be calibrated before each measurement using standardised gases. All testing will be conducted in the morning after a minimum 10 hour overnight fast. Testing will be performed in a thermo-neutral environment with participants lying supine in a comfortable position, head on a pillow, and a transparent ventilated hood placed over their head. During the measurement period, participants will be asked to remain as relaxed as possible without falling asleep and instructed not to talk or fidget. Oxygen consumption (VO_2) and carbon dioxide production (VCO_2) will be measured continuously for 30min. After discarding the first 10 min of data, REE will be calculated as the lowest consecutive 10 min average value, provided that the coefficient of variation within that 10 min interval is <5%. Resting energy expenditure will be calculated using the Weir equation [metabolic rate (kcal per day) = 1.44 (3.94 VO_2 + 1.11 VCO_2)] (70-72).

Doubly Labelled Water

Total daily energy expenditure during free-living conditions over 14 days (see Table 2) will be quantified using the criterion doubly labelled water (DLW) technique at baseline, 3 months and 9 months in a subsample of participants (n=24 total, 12 per group). Each participant will be asked if they would like to participate in doubly labelled water testing until the required number of participants is achieved. On each occasion, participants will be provided with a dose of isotope labelled water (10 atom% oxygen 18 (^{18}O) and 99.9 atom% deuterium (^{2}H)) with the dose based on body mass (1.35 g of DLW × body mass in kg). Participants will be asked to collect urine specimens daily over a 2-week period. Samples will be analysed by isotope ratio mass spectrometry (IRMS). Total daily energy expenditure (kJ) over the 2-week period will be divided by 14 to estimate mean total daily energy expenditure (73, 74).

Blood Pressure

At baseline, 3 months and 9 months, seated blood pressure will be recorded in a controlled environment using an automated sphygmomanometer and appropriately sized cuffs after a 5-minute quiet rest according to JNC 7 guidelines (75). The same arm will be used for all assessment visits with the appropriately sized cuff. Four consecutive readings will be recorded at ~2 min intervals with the mean of the last three measurements used for analysis.

Flash Glucose Monitoring

Flash glucose monitoring will be used to assess dynamic changes in glucose over a 2 week period at baseline and at the end of both the weight loss and weight maintenance periods (FreeStyle Libre Flash Glucose Monitoring System) (76). Participants will wear a sensor on the back of their upper arm for up to 14 days and have a reader to scan the sensor every 6-8 hours. This system measures interstitial glucose concentrations and continuously stores measurement values every 15 minutes, which will provide information about postprandial glucose responses as well as changes in glucose regulation during weight loss. Average interstitial glucose and AUC (77) will be calculated and evaluated at baseline, 3 months and 9 months.

Dietary analysis

Adherence to energy-restricted diets will be assessed using 3 x 24-hour dietary recalls (via phone, at random times) during the weight loss and weight maintenance phases. Participants will be asked to complete a 4-day weighed food record in the week prior to baseline appointments, in the last week of weight loss and the last week of weight maintenance phases. Participants will be asked to record all foods and drinks consumed during this time and to record weights or estimate volumes using standard measures where possible and provide as much detail as possible about branded products. If required, we will lend participants a set of kitchen food scales.

Data will be collected on non-consecutive days and entered into Foodworks Nutritional Analysis Software version 8 (Xyris Software, Brisbane, QLD, Australia) for analysis of macronutrient and micronutrient intake as well as total energy intake.

Study Food Liking and Palpability Scores

A liking score for almonds and the carbohydrate-rich snack foods will be assessed using a Labelled Affect Magnitude Scale (78) and a food action rating scale (79) which will rate foods for overall liking, liking of textures and liking of flavours. These tests will occur at screening and at the end of weight loss and weight maintenance phase to determine any change following long-term consumption of the test foods.

Eating Behaviour, Mood and Personality

Eating behaviour, mood and personality will be assessed at baseline and at the end of the weight loss and weight maintenance phases. Change over time will be assessed using a series of validated questionnaires. Eating behaviour via; Power of Food (80), Food Craving Scale (81), Eating Attitude Test (82), Pickiness/Fickiness Questionnaire (83), EAT-26 (82), Control of Eating Questionnaire (84). Mood and personality via; Profile of Mood States (85), Perceived Stress Scale (86), Zung Self-Rating Scale (87), Eysenck Personality Questionnaire (88), and Brief Sensation Seeking Scale (89).

Quality of Life, Functional Mobility and Pain

The Timed Up and Go (TUG) test is a test of functional mobility. This test requires participants to be timed while getting up, walking 3 meters, turning, returning to the chair, and sitting down again (90). Previous studies in adults have reported the CV error was 6% for the TUG test (91). The short-form 36 (SF36) questionnaire will be used for assessing overall quality of life, and pain will be assessed with the SF36 bodily pain subscale as well as a VAS scale to rate the intensity of pain at each major chronic and/or acute pain site. The nature of pain (at each site) will be rated using a short-form of the McGill pain questionnaire (92). All pain measures have been shown to be reliable and valid in adults (93), with the psychometrics of the SF-36 specific to the Australian population (94, 95).

Faecal Microbiota

Stool samples will be collected at baseline and following the weight loss and weight maintenance phases using OMNIgene GUT DNA Stabilization Kits (DNA Genotek). DNA extraction will be performed using MoBio Powerlyzer Powersoil DNA Isolation Kit (MoBio Laboratories, Carlsbad, California) as described previously (96). DNA concentration will be quantified fluorometrically with a Qubit dsDNA HS Assay kit (Life

Technologies). Faecal microbial composition will be assessed by 16S rRNA amplicon sequencing (97). This analysis will include determination of Firmicutes:Bacteroidetes ratios and quantification of *Akkermansia muciniphila* abundance. A subsample of highest responders (n=10) and lowest responders (n=10) (based on weight regain) will also undergo more detailed analysis of samples from pre-baseline, end of weight loss and end of weight maintenance. Samples will be assessed for identification of responder/non-responder phylogenetic and functional traits using shotgun metagenomic sequencing (98).

Liver Health

The amount of fat stored within the liver will be assessed using ultrasound. Participants will have their livers imaged using a clinical ultrasound scanner (Philips iU22 Ultrasound imaging system, Bothell, WA, USA) equipped with a 5-1 MHz high-resolution curved array transducer (Model c5-1, Philips). The amount of fat stored within the liver will be assessed using three different methods. Firstly, the size of the liver will be determined using the technique by Childs et al (99). Three linear measurements of the liver will be taken and then volume calculated by using the following equation: liver volume (cm^3) = 343.71 + [0.84 × ABC], where ABC is the product of the three linear measurements (99). Secondly, a visual assessment of fat accumulation will be evaluated according to the technique by Ballestri et al, adapted to liver images (100). A fatty liver indicator score ranging from 0 to 9 will be calculated using three indicators: (1) presence or absence of liver-kidney contrast; (2) assessment of beam penetration and (3) level of vessel wall blurring. Each measure will be graded as normal (score of 0), mild (score of 1), moderate (score of 2), or severe (score of 3) (100). Finally, quantification of liver fibrosis, a consequence of fatty liver, will be performed using shear wave elastography. The ultrasound machine emits a low-frequency pulse and software inbuilt into the ultrasound machine indicates the density of the liver tissue. The more fibrotic the tissue, the denser the tissue and the higher the reading (101).

Data Management

All participants will be given a unique code, which will be used to identify their electronic and paper-based data and biological samples. A password-protected computer database, accessible by the researchers only, will store participant identifiers (e.g., name, email address, phone number) and their associated code. Paper-based data will be stored securely at the University of South Australia for 15 years, after which it may be destroyed. Paper case report forms will be used to collect data at all clinic visits. Data will be entered twice into two separate password-protected Excel data files. Before analysis, data will be compared between files to ensure it has been correctly recorded. Biological samples will be stored in a swipe card secured −80°C freezer, with an alarm system that alerts a staff member if the temperature rises above a predetermined temperature. Samples will be stored for up to 15 years from collection date and disposed of accordingly after that time.

Protocol deviations

Deviations from the proposed protocol will be communicated via an update of the Australian and New Zealand Clinical Trial Registry and also through a letter to the editor of this journal.

Adverse Events

Adverse events will be recorded in the case report form and will be reported to the University of South Australia Human Research Ethics Committee. Adverse events that lead to withdrawals will be reported in future publications. We do not intend to formally analyse adverse events.

Statistical analysis plan

Statistical analysis will be performed using SPSS for Windows 24.0 (SPSS, Chicago, IL, USA). Data will be tested for normality, and where possible non-normally distributed data will be log transformed prior to analysis. The effects of diet treatment on the dependent measures over time will be analysed using random effects mixed models, with treatment (AED or NFD) and time (baseline, end of weight loss, and end of weight maintenance) being the between and within factors respectively in the analysis. Both

intention-to-treat (ITT) and per-protocol analyses (for those who achieve a minimum of 80% compliance with test food consumption) will be completed. Where main effects are identified, Bonferroni post hoc tests will be performed to identify significant differences between means (P set at <0·05). Whilst the ITT analysis will be the main analysis, the per-protocol analysis will allow us to decipher that the effects are due to participants being compliant with consuming test foods. We will also run sub-analyses for both the ITT and per-protocol analyses which will stratify participants according to weight loss during the weight loss phase to determine whether there is an interaction between dietary treatment and weight loss in terms of effects on the various outcomes. This will allow for determination of whether almonds, compared with control, provide greater improvements in outcomes for any given level of weight loss.

Data Access

There are no contractual agreements that require the data from this trial to be shared.

Ethics and Dissemination

Ethics approval was obtained from the University of South Australia Human Research Ethics Committee (201436).

Participants will receive a copy of their individual results (with the exception of liver scans, faecal sample testing, resting energy expenditure, doubly labelled water and some blood analysis results) as well as a summary of the study findings. Participants who complete all aspects of the study will receive an honorarium of $400 to compensate for their time and travel expenses. The subsample of participants who also participate in doubly labelled water assessments will be provided with an additional $150 honorarium. Findings of the study will be disseminated at scientific conferences and published papers. Further, where appropriate outcomes will be made available to the public via media releases.

Trial Registration

The protocol for this study was registered with the Australian New Zealand Clinical Trials Registry (ACTRN12618001861246).

DISCUSSION

Nut consumption in many countries is low, and it has been suggested that people may avoid eating nuts as they may perceive that the high-fat content will lead to weight gain (12, 13, 15, 16). However, various mechanisms have been hypothesized regarding the association between nut consumption and weight loss success. Nuts are rich in protein, fibre and monounsaturated fat, macronutrients known to have a positive effect on appetite control (25). The high unsaturated fat content and protein in nuts may lead to an increase in resting energy expenditure and diet-induced thermogenesis (31). Additionally, the structure of the lipid-storing granules and high fibre content of nuts, as well as incomplete mastication may cause reduced fat absorption resulting in a loss of available energy (31). Despite this, clinical trials evaluating the effects of nut consumption compared to a nut-free diet on adiposity have reported mixed findings, with some studies reporting greater weight loss (17, 18) or improved body composition (19, 20), and others reporting no weight change (1, 5, 9), suggesting further investigation is required.

Strengths

There are a number of novel features in this study design. To our knowledge, this will be the first trial to assess whether the inclusion of 15% of energy from almonds in an otherwise nut-free diet will improve weight loss and limit weight regain. As such, this work has the potential to expand the current understanding of how regular consumption of almonds can aid weight loss and weight maintenance, while also providing beneficial cardiometabolic, liver and gut health effects. Furthermore, the metagenomic analysis that will be performed will be a substantial advance on our current understanding of the impact of almonds on the gut microbiome (previously limited to amplicon sequencing approaches) (102).

Limitations

One of the main challenges with any dietary intervention study is recruiting participants and keeping them motivated. We have previously successfully recruited large numbers of participants for similar intervention trials and plan to support participants with regular appointments with the dietitian, as well as the provision of test foods and simple food checklists to assist with compliance. A potential limitation

of this study is that it will only be feasible to follow participants for 6 months after initial weight loss, although data from our recent pilot study suggests that this will be sufficient to observe differential changes in weight regain due to the rapidity of weight regain once intensive dietary support is removed (Wilson AL et al. Nudging Weight Loss Maintenance in Adults with Type-2 Diabetes: A Pilot Intervention).

This 9-month randomised controlled trial will add to the evidence base strategies to facilitate weight loss and prevent regain, potentially leading to recommendations to frequently substitute energy-dense snacks that lack nutritional value with nuts, which, in turn, could lead to improved weight loss outcomes and facilitate beneficial dietary habits (13, 103).

Author contributions: A.M.C, J.D.B, A.M.H, S.Y.T, G.B.R : were co-applicants on the grant application and as such were involved with the original design. A.M.C: was the lead applicant and is the principal investigator for the study. A.M.C, J.D.B, A.M.H, S.C, C.Y: are involved with study coordination and responsible for the day to day running of the trial, recruitment and sample collection. All authors (S.C, A.M.H, C. Y, J.D.B, S.Y.T, G.B.R, J.C, M.M, K.L, S.W, T.R.S, F.F, A.P.H, A.M.C): contributed to method development and the writing and development of the protocol paper and all authors (as above) will have responsibility for analysis, statistical interpretation of outcomes and preparation of manuscripts for publication post-study completion.

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Conflicts of interest: This study was funded by the Almond Board of California. A.M.C has consulted for Nuts for Life (an initiative of the Australian Tree Nut Industry).

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Figure Legend

Table 1. Eligibility Criteria

Table 2. Outcome Measures at Each Time Point

Table 3. Macronutrient Composition of Test Foods

Table 4. Blood and Faecal Analysis

Figure 1. Study Timeline

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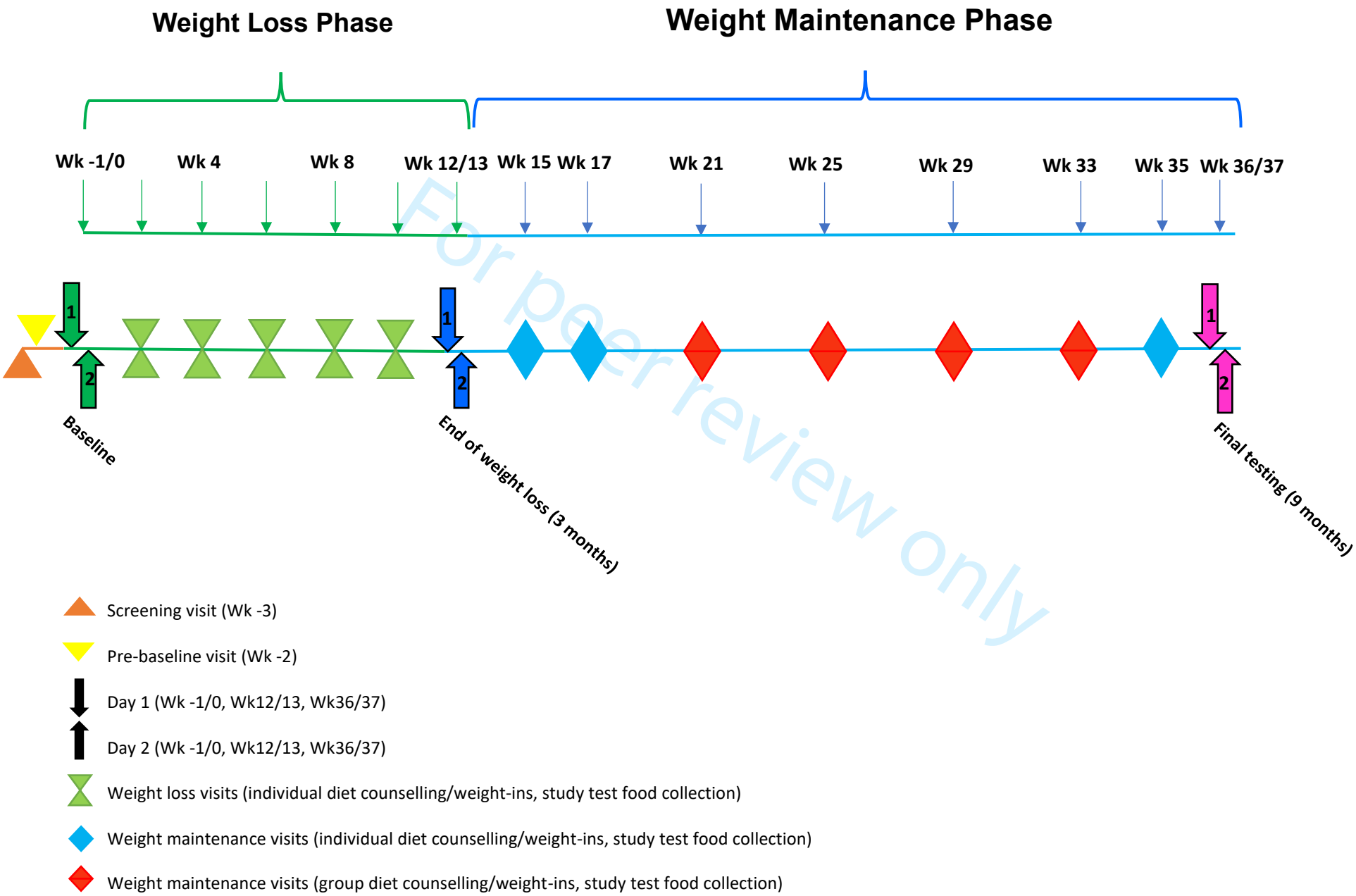
Figure 1: Study Timeline



SPIRIT 2013 Checklist: Recommended items to address in a clinical trial protocol and related documents*

Section/item	Item No	Description	Addressed on page number
Administrative information			
Title	1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym	1
Trial registration	2a	Trial identifier and registry name. If not yet registered, name of intended registry	1
	2b	All items from the World Health Organization Trial Registration Data Set	N/A
Protocol version	3	Date and version identifier	1
Funding	4	Sources and types of financial, material, and other support	28
Roles and responsibilities	5a	Names, affiliations, and roles of protocol contributors	1
	5b	Name and contact information for the trial sponsor	1
	5c	Role of study sponsor and funders, if any, in study design; collection, management, analysis, and interpretation of data; writing of the report; and the decision to submit the report for publication, including whether they will have ultimate authority over any of these activities	28
	5d	Composition, roles, and responsibilities of the coordinating centre, steering committee, endpoint adjudication committee, data management team, and other individuals or groups overseeing the trial, if applicable (see Item 21a for data monitoring committee)	28

Introduction			
Background and rationale	6a	Description of research question and justification for undertaking the trial, including summary of relevant studies (published and unpublished) examining benefits and harms for each intervention	3-5
	6b	Explanation for choice of comparators	12
Objectives	7	Specific objectives or hypotheses	4-5
Trial design	8	Description of trial design including type of trial (eg, parallel group, crossover, factorial, single group), allocation ratio, and framework (eg, superiority, equivalence, noninferiority, exploratory)	5-7
Methods: Participants, interventions, and outcomes			
Study setting	9	Description of study settings (eg, community clinic, academic hospital) and list of countries where data will be collected. Reference to where list of study sites can be obtained	5
Eligibility criteria	10	Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and individuals who will perform the interventions (eg, surgeons, psychotherapists)	6
Interventions	11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered	12-13
	11b	Criteria for discontinuing or modifying allocated interventions for a given trial participant (eg, drug dose change in response to harms, participant request, or improving/worsening disease)	N/A
	11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return, laboratory tests)	12
	11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial	N/A
Outcomes	12	Primary, secondary, and other outcomes, including the specific measurement variable (eg, systolic blood pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg, median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen efficacy and harm outcomes is strongly recommended	13-24
Participant timeline	13	Time schedule of enrolment, interventions (including any run-ins and washouts), assessments, and visits for participants. A schematic diagram is highly recommended (see Figure)	9-11

Sample size	14	Estimated number of participants needed to achieve study objectives and how it was determined, including clinical and statistical assumptions supporting any sample size calculations	7
Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size	6-7
Methods: Assignment of interventions (for controlled trials)			
Allocation:			
Sequence generation	16a	Method of generating the allocation sequence (eg, computer-generated random numbers), and list of any factors for stratification. To reduce predictability of a random sequence, details of any planned restriction (eg, blocking) should be provided in a separate document that is unavailable to those who enrol participants or assign interventions	7
Allocation concealment mechanism	16b	Mechanism of implementing the allocation sequence (eg, central telephone; sequentially numbered, opaque, sealed envelopes), describing any steps to conceal the sequence until interventions are assigned	7
Implementation	16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions	7
Blinding (masking)	17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how	7
	17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial	7
Methods: Data collection, management, and analysis			
Data collection methods	18a	Plans for assessment and collection of outcome, baseline, and other trial data, including any related processes to promote data quality (eg, duplicate measurements, training of assessors) and a description of study instruments (eg, questionnaires, laboratory tests) along with their reliability and validity, if known. Reference to where data collection forms can be found, if not in the protocol	9-11, 13-24
	18b	Plans to promote participant retention and complete follow-up, including list of any outcome data to be collected for participants who discontinue or deviate from intervention protocols	6

Data management	19	Plans for data entry, coding, security, and storage, including any related processes to promote data quality (eg, double data entry; range checks for data values). Reference to where details of data management procedures can be found, if not in the protocol	24-25
Statistical methods	20a	Statistical methods for analysing primary and secondary outcomes. Reference to where other details of the statistical analysis plan can be found, if not in the protocol	25
	20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	25
	20c	Definition of analysis population relating to protocol non-adherence (eg, as randomised analysis), and any statistical methods to handle missing data (eg, multiple imputation)	25
Methods: Monitoring			
Data monitoring	21a	Composition of data monitoring committee (DMC); summary of its role and reporting structure; statement of whether it is independent from the sponsor and competing interests; and reference to where further details about its charter can be found, if not in the protocol. Alternatively, an explanation of why a DMC is not needed	N/A
	21b	Description of any interim analyses and stopping guidelines, including who will have access to these interim results and make the final decision to terminate the trial	N/A
Harms	22	Plans for collecting, assessing, reporting, and managing solicited and spontaneously reported adverse events and other unintended effects of trial interventions or trial conduct	25
Auditing	23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor	N/A
Ethics and dissemination			
Research ethics approval	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval	2
Protocol amendments	25	Plans for communicating important protocol modifications (eg, changes to eligibility criteria, outcomes, analyses) to relevant parties (eg, investigators, REC/IRBs, trial participants, trial registries, journals, regulators)	2 & 25

Consent or assent	26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)	7
	26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	N/A
Confidentiality	27	How personal information about potential and enrolled participants will be collected, shared, and maintained in order to protect confidentiality before, during, and after the trial	24
Declaration of interests	28	Financial and other competing interests for principal investigators for the overall trial and each study site	28
Access to data	29	Statement of who will have access to the final trial dataset, and disclosure of contractual agreements that limit such access for investigators	24
Ancillary and post-trial care	30	Provisions, if any, for ancillary and post-trial care, and for compensation to those who suffer harm from trial participation	N/A
Dissemination policy	31a	Plans for investigators and sponsor to communicate trial results to participants, healthcare professionals, the public, and other relevant groups (eg, via publication, reporting in results databases, or other data sharing arrangements), including any publication restrictions	26
	31b	Authorship eligibility guidelines and any intended use of professional writers	28
	31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	26
Appendices			
Informed consent materials	32	Model consent form and other related documentation given to participants and authorised surrogates	Yes, sent to editor
Biological specimens	33	Plans for collection, laboratory evaluation, and storage of biological specimens for genetic or molecular analysis in the current trial and for future use in ancillary studies, if applicable	24

*It is strongly recommended that this checklist be read in conjunction with the SPIRIT 2013 Explanation & Elaboration for important clarification on the items. Amendments to the protocol should be tracked and dated. The SPIRIT checklist is copyrighted by the SPIRIT Group under the Creative Commons "Attribution-NonCommercial-NoDerivs 3.0 Unported" license.

BMJ Open

Study protocol for a 9-month randomised controlled trial assessing the effects of almonds vs. carbohydrate-rich snack foods on weight loss and weight maintenance

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Study protocol for a 9-month randomised controlled trial assessing the effects of almonds vs. carbohydrate-rich snack foods on weight loss and weight maintenance.

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Word Count (not including abstract): 6505 words

Strengths and limitations of the study:

- To our knowledge, this will be the first randomised controlled trial to assess whether the inclusion of almonds vs. carbohydrate-rich snack foods in an otherwise nut-free diet will improve weight loss and limit weight regain.
- A wide range of outcomes will be assessed including but not limited to; body composition, resting and total daily energy expenditure, appetite regulation, cardiometabolic health, liver health, inflammatory markers and effects on the gut microbiome.
- Both objective and subjective appetite regulation will be evaluated, adding to our current limited knowledge of the effects of almonds on appetite control.
- The metagenomic analysis that will be performed will be a substantial advance on our current understanding of the impact of almonds on the gut microbiome (previously limited to amplicon sequencing approaches).
- A potential limitation of this study is that it will only be feasible to follow participants for 6 months after initial weight loss.

ABSTRACT

Introduction Epidemiological studies indicate an inverse association between nut consumption and body mass index (BMI). However, clinical trials evaluating the effects of nut consumption compared to a nut-free diet on adiposity have reported mixed findings with some studies reporting greater weight loss and others reporting no weight change. This paper describes the rationale and detailed protocol for a randomised controlled trial assessing whether the inclusion of almonds or carbohydrate-rich snacks in an otherwise nut-free energy-restricted diet will promote weight loss during 3 months of energy restriction and limit weight regain during 6 months of weight maintenance.

Methods and analysis One hundred and thirty-four adults aged 25-65 years with a BMI of 27.5-34.9kg/m^2 will be recruited and randomly allocated to either the almond-enriched diet (AED) [15% energy from almonds] or a nut-free control diet (NFD) [15% energy from carbohydrate-rich snack foods]. Study snack foods will be provided. Weight loss will be achieved through a 30% energy restriction over 3 months, and weight maintenance will be encouraged for 6 months by increasing overall energy intake by ~120-180 kcal/day as required. Food will be self-selected, based on recommendations from the study dietitian. Body composition, resting energy expenditure, total daily energy expenditure (via doubly labelled water), physical activity, appetite regulation, cardiometabolic health, gut microbiome, liver health, inflammatory factors, eating behaviours, mood and personality, functional mobility and pain, quality of life and sleep patterns will be measured throughout the 9-month trial. The effects of intervention on the outcome measures over time will be analysed using random effects mixed models, with treatment (AED or NFD) and time (baseline, 3 months, and 9 months) being the between and within factors respectively in the analysis.

Ethics and dissemination Ethics approval was obtained from the University of South Australia Human Research Ethics Committee (201436). Results from this trial will be disseminated through publication in peer-reviewed journals, national and international presentations.

Trial registration number ACTRN12618001861246; Pre-results.

INTRODUCTION

Epidemiological studies report associations between increased frequency of nut consumption and lower body weight (1-4). This is supported by clinical data which suggest that regular nut intake has a beneficial impact on adiposity, insulin resistance and related metabolic abnormalities (5-11). Despite this, many people still avoid eating nuts due to the perception that they lead to weight gain based on their high energy and fat content. Nut consumption in many countries including Australia and America is low, (6 g/day and 3.3 g/day per capita, respectively) (12-14) with the prevalence of adult nut consumers being ~ 16-20% in Australia (15) and America (16). Such low consumption suggests there is scope to increase consumption in both countries.

Data from the National Health and Nutrition Examination Survey indicated that nut consumption was associated with a lower body mass index (BMI) (27.7 ± 0.2 kg/m^2 vs 28.1 ± 0.1 kg/m^2, $p < 0.05$) and waist circumference (95.6 ± 0.4 cm vs 96.4 ± 0.3 cm, $p < 0.05$), and tree nut consumers had lower body weight than non-consumers (78.8 ± 0.7 kg vs 80.7 ± 0.3 kg, $p < 0.05$) (16). When considering almonds specifically, randomised controlled trials have reported greater weight loss (17, 18) or improved body composition (reduced total fat and truncal fat) (19, 20) on hypocaloric diets with the inclusion of almonds compared to a nut-free diet. However, recent meta-analyses of clinical trials evaluating the effects of nut consumption on adiposity have reported no difference in body weight, BMI or waist circumference when comparing diets including nuts against control diets (5, 9). Nevertheless, changes in body fat distribution and reductions in fat stored in the liver can improve metabolic outcomes independent of weight changes. Inclusion of nuts may help prevent and manage non-alcoholic fatty liver disease (21, 22) and almond consumption has been associated with reductions in circulating liver enzyme concentrations (23). Additionally, data from large cohort studies suggest that frequent nut consumption may lower the risk of weight gain (4), with consumption on 5 or more days per week showing the greatest effect (24, 25). Prospective analysis of cohorts of healthy adults show that the average weight gain over 4 years was 3.3 lb (~1.5 kg). This weight gain was inversely associated with nut consumption (26).

Weight regain following initial weight loss is common and contributes, in part, to the obesity epidemic (27). Randomised controlled trials assessing weight loss maintenance report a 30-35% weight regain in the first year following a weight loss

intervention with a 76% weight regain at 4 years post-treatment (28). Weight regain suggests there is a need for nutritional strategies that prevent weight regain. Therefore assessing the effects of nutritional strategies, such as the inclusion of nuts for the prevention of weight regain, is paramount. Diets high in protein appear to limit weight regain (29) with higher intakes of non-cereal plant proteins, such as in nuts, associated with a protective effect (30). Nuts are rich in protein, fibre and monounsaturated fat, which have been suggested to contribute to their positive effect on appetite regulation (31, 32) and almonds, specifically, have been shown to have positive effects on subjective ratings of appetite (33, 34). However, the majority of assessments of appetite regulation with nuts have been conducted acutely and subjectively, and few have assessed appetite hormones (35). Reduced food cravings have been associated with long-term weight loss success (36); thus, assessing both of these outcomes following regular almond consumption is important.

Previous studies have reported significant differences in gut microbiota between lean and obese individuals (37) and modulation at the phylum and genus levels following weight loss (38). Changes in gut microbiota have also been observed following consumption of diets containing almonds (39) and almond skins (40), although not consistently in all populations (41). Significant increases in the relative abundance of bacterial taxa comprising of Ruminiclostridium, and members of the Ruminococcaceae and Lachnospiraceae families, under trial conditions are implicated in the degradation of complex dietary plant-derived polysaccharides, and the production of beneficial short-chain fatty acids (42). It has been suggested that bacterial products may impact on short-term intestinal satiety pathways and long-term appetite control, acting directly on hypothalamic neurons (43). However, it is unclear whether the changes in gut microbiota associated with almond consumption may help limit weight regain and the relationship with other biomarkers of cardiometabolic risk.

OBJECTIVES

Primary objective

The primary aim of this project is to evaluate whether inclusion of 15% of energy from almonds [almond-enriched diet (AED)] compared to carbohydrate-rich snacks in

an otherwise nut-free energy-restricted diet [nut-free control diet (NFD)], will improve weight loss during 3 months of dietary energy restriction and limit weight regain during 6 months of weight maintenance. We hypothesize that the AED will lead to greater weight loss during the energy restriction phase of 3 months and limit weight regain during the weight maintenance period of 6 months compared to the NFD.

Secondary objectives

The secondary aim is to evaluate whether an AED compared to a NFD improves body composition and body fat distribution (reduced waist circumference and abdominal fat depots), impacts resting and total daily energy expenditure and improves subjective and objective measures of satiety.

Tertiary objectives

The tertiary aim is to evaluate whether an AED compared to a NFD reduces fat accumulation in the liver and improves liver enzyme profiles, results in beneficial changes in the composition of the gut microbiome, improves inflammatory biomarkers and cardiometabolic health outcomes including blood lipid profiles, glucose and insulin, and improves self-reported eating behaviours, mood, personality, pain, functional mobility, quality of life, sleep and physical activity patterns.

METHODS AND ANALYSIS

Study design

The study is designed as a 9-month randomised controlled parallel-arm dietary intervention. The study will be conducted in the research facilities of the Alliance for Research in Exercise, Nutrition and Activity (ARENA) at the University of South Australia, Adelaide. The SPIRIT guidelines were used in the development of this protocol (44).

Patient and public involvement

Development of this research protocol was done without patient involvement. The final study results will be disseminated to all participants.

Participants

Eligibility criteria

Participants will be male and female volunteers, aged 25-65 years, with a BMI of 27.5-34.9 kg/m^2. This age range ensures physical maturity has been achieved and limits the possibility of chronic health conditions that would exclude the volunteer from participation. This BMI range ensures sufficient weight available to lose and reduces the risk of chronic health conditions. Participants will be non-smokers (minimum 6 months) and weight stable (within 5 kg) for 3 months prior to enrolment. Detailed inclusion and exclusion and withdrawal criteria are listed in table 1.

Table 1. Eligibility Criteria

Inclusion Criteria
Males and females aged 25-65 years
Body mass index (BMI) 27.5-34.9 kg/m^2
Weight stable (within 5kg) in the past 3 months
Non-smoker (minimum 6 months)
Exclusion Criteria
Cardiovascular Disease
Type 1 or Type 2 Diabetes
Thyroid Disorders
Kidney or Liver disease
Gastrointestinal disorders requiring medical nutrition therapy (e.g. Crohn's disease, irritable bowel, coeliac disease)
Are pregnant or breastfeeding
Allergies to nuts, gluten or other components of the test foods
Unable to chew hard foods such as nuts
Consumed more than 30g of nuts per week in the month prior to beginning the trial
Alcohol (>14 standard drinks/week) (1 standard drink = 100 ml wine, 285 ml beer, 30 ml spirit) or drug dependency
Have changed medications or supplements in the last 3 months
Take vitamin, mineral, herbal supplementation or medications that may have an impact on study outcomes
Unwilling to stop dietary supplements that influence weight
Suffer claustrophobia or a fear of enclosed spaces
Show unwillingness to be randomised to either experimental group (based on liking and palatability questionnaires)
Withdrawal Criteria
Adverse reaction to test foods
The need to take a medication or treatment, which in the opinion of the investigator, may interfere with study measurements
Consistent non-compliance (<80% compliance) with consuming study foods.
Failure to satisfy the investigator regarding suitability to participate for any other reason

Recruitment/screening

Participants will be recruited from the public through radio, TV, printed media, internet-based advertisements and flyer distribution. Procedures will occur in accordance with ethical standards, including obtaining written informed consent.

Interested participants will be sent the participant information and a diet and lifestyle questionnaire (DLQ) to determine eligibility. Participants who appear eligible from DLQ responses will be assigned a screening number and will undergo an initial screening interview over the telephone to review medical history, concomitant medication and supplementation. Prospective participants will attend the clinical research facility approximately 3 weeks before baseline where eligibility will be confirmed, and the likelihood to consume test foods will be established via liking and palatability questionnaires (see Table 2). Participants who meet the required criteria and are deemed eligible, will be asked to provide written informed consent in the presence of the investigator and will proceed with a pre-baseline clinic appointment, 2 weeks prior to baseline.

Randomisation, allocation concealment and sequence generation

Data collected at the screening visit will be used to assign participants to groups based on age, sex, and BMI in the process of randomisation by minimization (45). Minimisation will ensure baseline characteristics are balanced between the treatment groups and has been proposed to be the most suitable randomisation method for small clinical trials, such as the proposed study, to reduce bias (45, 46). A staff member independent of the study outcome assessments and statistical analysis will perform the treatment allocation and maintain the randomisation list in a secure location with access limited to authorised personnel. As the participants are consuming whole foods, which are easily identified, the participants and staff involved in diet management cannot be blinded. Staff conducting clinical assessments at baseline, 3 months and 9 months will remain blinded to treatment group allocation. Participants will be asked not to disclose the foods they are consuming to the researchers. Researchers conducting assessments and analysing data will remain blinded until the completion of statistical analysis.

Sample size calculation

The study is powered on the primary outcomes of weight loss and weight regain. One hundred participants will provide 80% power to detect a 2.4 kg difference in weight loss (18) and a 1.7 kg difference in weight regain (based on variance in our pilot data) (Wilson AL et al. Nudging Weight Loss Maintenance in Adults with Type-2 Diabetes: A Pilot Intervention) between the two groups (α-level of 0.05). One hundred and thirty-four participants will be recruited (n=67 in each group) to allow for a 25% dropout.

Pre-intervention

Two weeks prior to baseline visits participants will be asked to attend a pre-baseline session. During this visit, a flash blood glucose monitoring sensor (FreeStyle Libre, Abbott, Australia) will be inserted for collection of blood glucose measures for 2 weeks, a wrist-worn accelerometer (GENEActiv, Activinsights Ltd, UK) will be provided for measuring physical activity and sleep patterns for 2 weeks, and a test kit will be provided for a single stool sample collection. Participants will be asked to keep a 4-day weighed food diary (non-consecutive days with 1 weekend day) and a 14-day sleep diary. Several questionnaires will also be administered to assess eating behaviour, mood, stress and personality (see Table 2).

Table 2. Outcome Measures at Each Time Point

	Screening	Pre-baseline	Baseline		Study Period				Close-out	
Time (Weeks) from Start of Dietary Intervention	**-3**	**-2**	**-1(D1)**	**0 (D2)**	**10**	**12 (D1)**	**13 (D2)**	**35**	**36 (D1)**	**37 (D2)**
Study Food Liking & Palatability Scores										
Labelled Affect Magnitude Scale	X								X	
Food Action Rating Scale	X								X	
Body Composition										
Height	X	X								
Weight (& Body Mass Index)	X	X	X[a]	X	X	X[a]	X	X	X[a]	X
Total and Truncal Fat Mass (DXA)			X			X			X	
Total and Truncal Fat-Free Mass (DXA)			X			X			X	
Visceral Adipose Tissue (DXA)			X			X			X	
Waist Circumference			X			X			X	
Energy Expenditure										
Accelerometry		X			X			X		
Resting Energy Expenditure (Indirect Calorimetry)			X			X			X	
Total Daily Energy Expenditure (Doubly Labelled Water)[b]		X				X			X	
International Physical Activity Questionnaire			X			X			X	
Appetite Regulation and Eating Behaviour										
Energy Intake										
4-day Food Diary		X			X			X		
24-hour Diet Recalls[c]							X			X
Fasting and Postprandial Gut Hormones & Glucose										
Glucagon-like peptide-1				X			X			X
Ghrelin				X			X			X
Leptin				X			X			X
Pancreatic Polypeptide				X			X			X

	Screening	Pre-baseline	Baseline		Study Period				Close-out	
Time (Weeks) from Start of Dietary Intervention	**-3**	**-2**	**-1(D1)**	**0 (D2)**	**10**	**12 (D1)**	**13 (D2)**	**35**	**36 (D1)**	**37 (D2)**
Glucose-Dependent Insulinotropic Polypeptide (Gastric Inhibitory Polypeptide)				X			X			X
Peptide Tyrosine Tyrosine (Peptide YY)				X			X			X
C-Peptide				X			X			X
Cholecystokinin (CCK)				X			X			X
Glucagon				X			X			X
Glucose				X			X			X
Drive to Eat and Eating Behaviour										
Subjective Drive to Eat (Visual Analogue Scales) – Fasting and Postprandial				X			X			X
Energy Consumed at a Buffet Meal				X			X			X
Power of Food Survey			X			X			X	
Food Craving Scale			X			X			X	
Pickiness/Finickiness Questionnaire			X						X	
Eating Attitude Test (EAT-26)			X			X			X	
Control of Eating Questionnaire			X			X			X	
Psychology and Health										
General Health, Pain, Mobility, Mood and Personality										
Short-form 36 (SF36) Questionnaire				X			X			X
Profile of Mood States (POMS)				X			X			X
Perceived Stress Scale		X				X			X	
Zung Self-Rating Scale		X				X			X	
McGill Pain Scale and Chronic & Acute Pain Scales (Visual Analogue Scales)				X			X			X
Timed Up and Go (Functional Mobility)			X			X			X	
Eysenck Personality Questionnaire		X								
Brief Sensation Seeking Scale		X								
Gut Health										
Faecal microbiome composition		X			X			X		
Liver Health										

	Screening	Pre-baseline	Baseline		Study Period				Close-out	
Time (Weeks) from Start of Dietary Intervention	**-3**	**-2**	**-1(D1)**	**0 (D2)**	**10**	**12 (D1)**	**13 (D2)**	**35**	**36 (D1)**	**37 (D2)**
Liver Ultrasound				X			X			X
Alanine Aminotransferase (ALT)			X			X			X	
Aspartate Aminotransferase (AST)			X			X			X	
Alkaline Phosphatase (ALP)			X			X			X	
γ-Glutamyltransferase (GGT)			X			X			X	
Cardiometabolic Health										
Blood pressure	X	X	X			X			X	
Flash Glucose Monitoring		X			X			X		
Insulin				X			X			X
HOMA				X			X			X
Triglycerides			X	X		X	X		X	X
Cholesterols (TC, HDL-C, LDL-C, VLDL-C, IDL-C, and subclasses, Lipoprotein (a), Oxidised LDL, LDL-C particle size)			X			X			X	
Apolipoprotein B			X			X			X	
Apolipoprotein A1			X			X			X	
Inflammatory Markers										
F2-Isoprostane levels (plasma + urine)			X			X			X	
C-reactive protein			X			X			X	
Adiponectin			X			X			X	
Sleep Patterns										
Pittsburg Sleep Quality Index			X			X			X	
14-day sleep diary		X			X			X		
Biomarkers of Compliance										
αlpha-tocopherol			X			X			X	

[a] Indicates primary outcome timepoints (fasted weight); [b] Sub-sample only; [c] 3 x 24-hour recalls were completed at random intervals between 0-13 weeks and 13-37 weeks; D1, day 1; D2, day 2; DXA, dual-energy X-ray absorptiometry; HDL-C, high density lipoprotein-cholesterol; IDL-C, intermediate density lipoprotein-cholesterol; LDL-C, low density lipoprotein-cholesterol; TC, total cholesterol; VLDL-C, very low density lipoprotein-cholesterol.

Study Intervention

The intervention will be a 9-month protocol, consisting of 3 months weight loss and 6 months weight maintenance. During the 9-month study period, participants in the AED will incorporate 15% of their energy as unsalted, whole, natural almonds with skins while participants in the NFD will include 15% of their energy consumed as carbohydrate-rich snack foods (oven-baked fruit cereal bar and rice crackers) as part of a 30% energy restricted weight loss diet. It is expected that the minimum quantity of almonds required to contribute 15% of energy will be 30 g, which is consistent with dietary guidelines (47). The control foods have been selected as they are commonly consumed snacks, and do not contain the beneficial micro and macro-nutrients available in almonds but can provide equal energy density (see Table 3). Participants will be provided with test foods to consume 6 days per week so that they have 1 day per week free from consuming test food. This has previously been found to support compliance (48). Checklists will be used to record daily consumption of study food and participants will be asked to return leftover food to calculate compliance scores. The threshold of compliance with test food consumption is >80%. All participants will be asked to avoid all other nuts and nut products during the entire study.

Table 3. Macronutrient Composition of Test Foods

Per 100g	Unsalted, Whole, Natural Almonds with Skin	Weight Watchers Apple Crumble Bar	Rice Crackers, White Rice, Other
Energy (kJ)	2385	1270	1646
Protein (g) (%)	19.7 (14.0)	4.4 (5.9)	9.4 (9.8)
Total fat (g) (%)	50.5 (78.3)	1.0 (2.9)	5.6 (12.5)
Saturated fat (g)	3.8	0.3	1.4
Polyunsaturated fat (g)	12.8	0.3	1.2
Monounsaturated fat (g)	30.7	0.3	2.6
Carbohydrate (g) (%)	5.4 (3.6)	55.7 (72.4)	74.6 (76.8)
Sugars (g)	5.2	27.5	1.7
Starch (g)	0.2	28.2	72.8
Fibre (g) (%)	10.9 (3.7)	14.7 (9.3)	1.8 (0.9)

Foods analysed in Foodworks Nutritional Analysis Software version 9 (Xyris Software, Brisbane, QLD, Australia).

Energy requirements will be determined by using the Schofield Equation, based on age, sex, and baseline body weight, as well as self-reported physical activity captured via the International Physical Activity Questionnaire (IPAQ) (49). Energy recommendation for weight loss will be 30% less than requirements to achieve 0.5-1 kg weight loss per week. Participants will be guided to consume a variety of foods within all five food groups to ensure they are still meeting nutrient reference guidelines consistent with Australian Dietary Guidelines (47). Participants will be provided with food group serve advice consistent with an energy restriction plan closest to their weight loss energy requirement of either 1200, 1500 or 1800 kcal/day (5000, 6300 or 7600 kJ/day). Weight loss plans will be adapted if more energy is required. We will lend participants a set of kitchen food scales to weigh food to ensure serve size accuracy. Three sample meal plans will be provided, as well as a recommended discretionary serve allowance of 2 per week, consistent with their energy requirement. Diet checklists will be used to assist with dietary compliance. During the weight maintenance phase, participants will be encouraged to stabilise

their weight by increasing their overall energy intake by ~120-180 kcal/day (~500-750 kJ/day), with additional adjustments as required through dietetic consultation.

Participants will attend two baseline appointments (Day 1, -1 week and Day 2, 0 weeks before intervention) and will be asked to refrain from alcohol for 24 hours and fast for a minimum of 10 hours prior to assessments on both days. Day 1 and Day 2 testing will occur again at 3 months and at 9 months. Details of tests, assessments and outcome measures completed on Days 1 and 2 (baseline, 3 months and 9 months) are available in Table 2. Participants will meet with the study dietitian at the end of the baseline Day 2 appointment to receive initial dietary counselling and instructions on test food consumption requirements. They will then meet individually with a dietitian every 2 weeks during the weight loss phase to have their weight monitored and test food compliance checked. During weight maintenance, participants will meet individually with the dietitian every 2 weeks for the first month and then monthly in small groups. Figure 1 outlines the study timeline. Adherence to energy-restricted diets will be assessed using 3 x 24-hour dietary recalls (via phone, at random times) during each phase, with weighed food diaries at baseline and at the end of the weight loss and weight maintenance phases. Participants will also be encouraged to meet national physical activity guidelines for Australian adults - 150 to 300 minutes (2 ½ to 5 hours) of moderate intensity physical activity or equivalent, per week (47). Accelerometer data, as well as self-reported activity via the IPAQ, will monitor whether 'unexpected' weight change might be explained by physical activity levels, and will assist in understanding the weight loss effects attributable to consuming almonds.

Data Collection

The following section outlines the data and biochemical samples being collected during the test periods (see Table 2 for a summary).

Anthropometry

Anthropometric assessments will be taken at Day 1 baseline, 3-month and 9-month appointments. All anthropometric assessments will be conducted with participants barefoot and wearing light clothing. Height will be measured twice to the nearest 1 mm with the average value calculated and recorded using a stadiometer at baseline

(SECA 216 Height Measuring Rod, SECA). Body weight will be recorded to the nearest 100 g following an overnight fast and will be measured twice on each occasion using calibrated electronic scales (TANITA Ultimate Scale 2000, Tanita Corporation, Tokyo, Japan) and the average value calculated. The same scales will be used throughout the intervention. BMI will be calculated as weight/height squared (kg/m2). Waist circumference will be measured, according to the protocol of the International Society for the Advancement of Kinanthropometry (50), using a metal measuring tape at the narrowest point of the abdomen or, if there is no obvious narrowing, at the midpoint between the lower costal (10th rib) border and the iliac crest. Two measurements will be taken unless they differ by more than 2% whereby a third measurement will be obtained. The mean of the measurements will be used for analysis. Body composition will be determined from a whole body dual-energy x-ray absorptiometry (DXA) scan, (Lunar ProdigyModel, GE Healthcare, Madison, WI, USA). Participants will wear a light disposable gown, and all external metal objects will be removed. Total body fat mass (%, kg), total body lean mass (%, kg), regional fat mass (arms, legs, trunk and abdominal fat mass, android, gynoid (kg)) and visceral adipose tissue amount and volume (kg and cm3) will be obtained using enCORE™ 2015 software (GE Healthcare enCORE version 13.31). VAT mass is converted to volume (cm3) at 1 g = 1.06 cm3 (GE, Europe). Precision estimates for iDXA measurements on the DXA scanner are 0.8% CV for total fat mass and 0.5% CV for VAT mass (51).

In addition to the measurements taken at baseline, at the end of weight loss and weight maintenance phases, non-fasting weight will also be measured during dietetic counselling appointments to provide feedback to participants. We will also lend participants scales (Withings/Nokia WBS06, Nokia) with Bluetooth capacity to enable them to monitor their weight at least twice per week at home and for these data to be sent to research staff to assist with weight monitoring. Regular weight monitoring has been shown to enhance success in weight loss (52) and weight maintenance trials (53).

Biochemical Measures

At Day 1 baseline, 3-month and 9-month appointments, fasting (>10 hours) venous blood samples will be taken by a trained phlebotomist. Collected blood samples will

be centrifuged (4° C, 4000 rpm, 10 mins) to separate plasma or serum and stored at −80°C for later analysis (see Table 4).

Table 4. Blood, Urine and Faecal Analysis

Parameter	Analysis Method	Sample Collected (Additives)
TC	Vertical Auto Profile (VAP II) (54)	Serum
HDL-C + subclass	Vertical Auto Profile (VAP II) (54)	Serum
LDL-C + subclass	Vertical Auto Profile (VAP II) (54)	Serum
IDL-C + subclass	Vertical Auto Profile (VAP II) (54)	Serum
VLDL-C + subclass	Vertical Auto Profile (VAP II) (54)	Serum
Lipoprotein(a)	Vertical Auto Profile (VAP II) (54)	Serum
Oxidised LDL-C	Solid phase 2-site ELISA	Serum
LDL particle number and size	Nuclear magnetic resonance (NMR) spectroscopy (55)	Serum
Triglyceride	Konelab Auto Analyser	Plasma
APOB	Vertical Auto Profile (VAP II) (54) and patented equations (20)	Serum
APOA1	Vertical Auto Profile (VAP II) (54) and patented equations (20)	Serum
hs-CRP	Konelab Auto Analyser	Serum
Adiponectin	ELISA	Serum
F2-Isoprostanes	Electron-capture negative-ion gas chromatography-mass spectrometry (56)	Plasma + Urine (Butylated Hydroxytoluene)
Alpha-tocopherol	High-performance liquid chromatography using the photo-diode array method (57)	Plasma
ALT	Abbott Alinity C	Serum
AST	Abbott Alinity C	Serum
ALP	Abbott Alinity C	Serum
GGT	Abbott Alinity C	Serum
Glucose	Konelab Auto Analyser	Plasma
Insulin	Mercodia ELISA	Plasma (Protease inhibitor and DPP-IV)
HOMA	Calculated using the Homeostasis Model Assessment Calculator v.2.3.3 (58)	-
Glucagon	Multiplex analysis system	Plasma (Protease inhibitor and DPP-IV)
GLP-1	Multiplex analysis system	Plasma

		(Protease inhibitor and DPP-IV)
Ghrelin	Multiplex analysis system	Plasma (Protease inhibitor and DPP-IV)
Leptin	Multiplex analysis system	Plasma (Protease inhibitor and DPP-IV)
Pancreatic Polypeptide	Multiplex analysis system	Plasma (Protease inhibitor and DPP-IV)
GIP	Multiplex analysis system	Plasma (Protease inhibitor and DPP-IV)
PYY	Multiplex analysis system	Plasma (Protease inhibitor and DPP-IV)
C-Peptide	Multiplex analysis system	Plasma (Protease inhibitor and DPP-IV)
CCK	ELISA (Ray Biotech)	Plasma (Protease inhibitor and DPP-IV)
Total Daily Energy Expenditure	Doubly Labelled Water	Urine
Faecal Microbiota	MoBio Powerlyzer Powersoil DNA Isolation Kit	OMNIgene GUT DNA Stabilization Kit

ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; APOA1, apolipoprotein A1; APOB, apolipoprotein B; CCK, cholecystokinin; DPP-IV, dipeptidyl peptidase-4; ELISA, enzyme-linked immunosorbent assay; GGT, γ-Glutamyltransferase; GLP-1, glucagon-like peptide-1; GIP, glucose-dependent insulinotropic polypeptide (gastric inhibitory polypeptide); HDL-C, high density lipoprotein-cholesterol; hs-CRP, high-sensitivity c-reactive protein; IDL-C, intermediate density lipoprotein-cholesterol; LDL-C, low density lipoprotein-cholesterol; NMR, nuclear magnetic resonance; PYY, peptide tyrosine tyrosine (peptide YY); TC, total cholesterol; VAP, vertical auto profile; VLDL-C, very low density lipoprotein-cholesterol.

Lipids

Serum lipids, cholesterol lipoprotein subclasses, and apolipoproteins will be assayed using Vertical Auto Profile (VAP II) (Atherotech Diagnostics Lab, Birmingham, AL), which directly measures cholesterol in all lipoprotein classes (54). Serum lipoprotein particle number and size will be assessed by a proton magnetic resonance spectroscopy assay (NMR LipoProfile III; LipoScience, Raleigh, NC), which measures the particle concentrations of lipoprotein subclasses and average particle size of lipoproteins (55). VAP II quantifies cholesterol concentrations of total lipoprotein, HDL, LDL, very-low-density lipoprotein (VLDL), lipoprotein(a) [Lp(a)], intermediate-density lipoprotein (IDL), and HDL, LDL, VLDL, and IDL subclasses. ApoB and ApoA1 will be calculated using results from the VAP test and patented equations (20). Serum oxidised LDL will be measured in duplicate by a solid phase 2-site ELISA (Mercodia, Uppsala, Sweden). Plasma triglyceride (%CV <5%) will be measured using a Konelab Auto Analyser.

Inflammatory Markers

Fasting plasma and a spot urine sample will be collected for analysis of F2-Isoprostanes as biomarkers of oxidative stress. Samples will be stabilised with butylated hydroxytoluene (BHT). Plasma and urine F2-Isoprostanes will be measured as total (free plus esterified) F2-Isoprostanes using electron-capture negative-ion gas chromatography–mass spectrometry as described previously (56). Serum will be collected for assessment of adiponectin by ELISA (Linco Research, St. Charles, Missouri) (59) and high-sensitivity C-reactive protein (hs-CRP) (%CV intra-assay 2.9%, inter-assay 1.9%) will be measured using a Konelab Auto Analyser.

Liver Enzymes

Fasting serum levels of alanine aminotransferase (ALT) (%CV intra-assay 0.5%, inter-assay 1.0%), aspartate aminotransferase (AST) (%CV intra-assay 0.6%, inter-assay 0.8%), alkaline phosphatase (ALP) (%CV intra-assay 0.3%, inter-assay 1.7%) and γ-glutamyltransferase (GGT) (%CV intra-assay 0.4%, inter-assay 1.1%) and will be measured using a local pathology service (23). To eliminate the effect of freeze-thawing of samples that may lower enzyme activity values, ALT, AST, ALP and GGT testing will be conducted on samples immediately transferred to the pathology laboratory.

Biomarkers of Compliance

Compliance with long-term almond consumption will be confirmed by measuring alpha-tocopherol levels (60, 61). Plasma alpha-tocopherol levels will be analysed using high-performance liquid chromatography with photo-diode array method according to Liu et al (57).

Appetite Regulation

At Day 2 baseline, 3-month and 9-month appointments, objective and subjective measures of appetite and satiety will be assessed. A test snack will be consumed following an overnight fast (>10 hours). The test snack for the AED group will comprise 15% of daily energy intake from almonds, and the NFD group will have 15% of daily energy intake from a high carbohydrate snack (oven-baked fruit filled bar). Blood samples will be obtained at time 0 (before the test snack) and every 30 minutes for 2 hours post the test snack, with participants asked to consume the test snack within 10 minutes. Blood samples will be collected via a BD Nexiva™ cannula blood collection system by a trained phlebotomist. Collected blood samples will be centrifuged (4° C, 4000 rpm, 10 mins) to separate plasma or serum and stored at −80°C for later analysis of gut hormones and glucose at all time points and triglycerides at time 0 minutes only (see Table 4). Both groups will be given 200mL of water to consume with their snack food. A further 300mL of water will be provided over the 2-hour testing period. Participants will be required to drink all water provided. After 2 hours, a buffet meal will be provided, and participants will be advised to eat as much or as little as they like within 30 minutes. The buffet meal will be free of nuts and will provide a selection of core and noncore foods and beverages as defined by the Australian Dietary Guidelines for participants to select (47). The foods chosen from the buffet meal will be assessed for total energy consumed, macronutrient and micronutrient composition using Foodworks Nutritional Analysis Software version 9 (Xyris Software, Brisbane, QLD, Australia).

A protease inhibitor cocktail [protease inhibitor (Sigma P2714) and DPP-IV (Millipore DPP4-010)] will be added immediately to the blood sample intended for testing; glucagon-like peptide-1 (GLP1), ghrelin, leptin, pancreatic polypeptide (PP), glucose-dependent insulinotropic polypeptide (GIP), peptide tyrosine tyrosine (PYY), cholecystokinin (CCK), C-peptide, insulin and glucagon. The samples will be

analysed using a multiplex analysis system (LUMINEX MAGPIX, ThermoScientific). CCK will be assessed using ELISA (Ray Biotech) (%CV intra-assay <10%, inter-assay <15%) and insulin will be assessed at baseline only using Mercodia ELISA (%CV <5%) and insulin resistance will be calculated using the Homeostasis Model Assessment (HOMA2) Calculator v2.2.3 82. Glucose and triglycerides will be analysed via Konelab Auto Analyser (%CV <5%).

Subjective ratings of hunger, appetite and fullness will be measured by visual analogue scales (VAS) at time 0 (before the test snack) and every 30 minutes for 2 hours post the test snack, as well as post buffet meal. VAS scales will be presented on separate sheets and recorded to the nearest mm. VAS responses will be recorded on a 100 mm line and measured to the nearest mm as the distance from the left hand anchor, with “not/none at all /no desire” on the left and “extremely/great desire” on the right. Questions will include “How hungry do you feel?”, “How thirsty do you feel?”, “How satisfied do you feel?”, “How full do you feel?” and “How much do you think you can eat?”. The validity and reliability of this approach have previously been established (62, 63). Completed VAS score sheets will be removed, so participants are not able to see their previous scores. While participants will not be blinded to the food consumed, the researchers evaluating VAS data will remain blinded. Area under the curve (AUC) for responses to VAS scales (mm) will be plotted over time and calculated for each satiety/hunger measure using the trapezoidal estimation method (64).

Accelerometry

Physical activity will be measured using triaxial accelerometers (GENEActiv Original, Activinsights Ltd, UK), which will be worn on the non-dominant wrist. Participants will be asked to wear the monitor 24 hours/day for 14 consecutive days at pre-baseline, and 2 weeks before the end of the weight loss and weight maintenance phases (see Table 2), removing it for showering/bathing or any other water-based activities. Devices will be configured through the manufacturer’s software (GENEActiv PC Software, Activinsights, UK) to record at 50 Hz for 14 days, starting at midnight of the first day of the monitoring period.

Participants will be provided with a paper-based record sheet to document; (1) the time they went to bed (“bedtime”), (2) the time they woke up (“get up time”) and (3)

the time the device was removed ("non-wear") and put back on again as well as the reason for removal (e.g., showing).

After the device is returned, the research team will download the raw acceleration data through the manufacturer's software. The Signal Vector Magnitude (SVM) of the acceleration, minus gravity, will be computed and summed over 60-second epochs:

$$SVM = \sum_{60s} \left| \sqrt{a_X^2 + a_Y^2 + a_Z^2} - g \right|$$ Where a_x, a_y, a_z are the three components of the acceleration signal and g the acceleration of gravity (9.81 m/s^2). The 60-second epoch data will then be imported into custom Matlab software for further processing. This software (*Cobra*, developed at the University of South Australia) provides a user-friendly graphical user interface for processing accelerometer data. Each 60-second epoch of waking wear time will be classified into one of four physical activity levels: sedentary, light, moderate or vigorous PA. Cutpoints for PA levels are defined according to Esliger et al. for adults (65) and adjusted proportionally to account for the 50 Hz sampling frequency (66). The resulting cutpoints between sedentary and light, light and moderate, and moderate and vigorous PA are 188, 403 and 1131 gravity units per minute (g.min), respectively. Device removals (non-wear) will be identified using the self-reported records and excluded from analysis. Where the reason given for removal is "sport", the removal period will be replaced with a period of moderate to vigorous physical actviity.

Sleep will be identified using the self-reported records. Sleep times will be corrected by visual inspection when necessary, that is, in case sleep times were not reported or when obvious discrepancies were observed between reported sleep and accelerometer trace. Sleep quality will be assessed through total sleep time and sleep fragmentation (67). Each minute between "bed time" and "get up time" will be classified as sleep or wake using the algorithm developed by van Hees et al. to detect wake periods during the night (68). Total sleep time is the sum of all sleep minutes between "bedtime" and "get up time". Sleep fragmentation is the ratio of total sleep time over time in bed.

All sleep and physical activity variables will be averaged over monitoring days for each participant. Averages over weekdays (Monday-Friday) and weekend days (Saturday, Sunday) will also be computed to assess any potential differences in physical activity between the two. A day will be considered invalid and excluded from

analysis if it included ≤10 hours wear during waking hours (69). A participant will be considered invalid and excluded from analysis if they provide <4 valid days of accelerometry data over the 14 day testing period on any testing occation (69, 70).

Resting Energy Expenditure

At Day 1 baseline, 3-month and 9-month appointments, resting energy expenditure (REE) will be measured using a ventilated hood system (TrueOne 2400 Metabolic System, ParvoMedics Inc, Sandy, UT, USA), which will be calibrated before each measurement using standardised gases. All testing will be conducted in the morning after a minimum 10 hour overnight fast. Testing will be performed in a thermo-neutral environment with participants lying supine in a comfortable position, head on a pillow, and a transparent ventilated hood placed over their head. During the measurement period, participants will be asked to remain as relaxed as possible without falling asleep and instructed not to talk or fidget. Oxygen consumption (VO_2) and carbon dioxide production (VCO_2) will be measured continuously for 30min. After discarding the first 10 min of data, REE will be calculated as the lowest consecutive 10 min average value, provided that the coefficient of variation within that 10 min interval is <5%. Resting energy expenditure will be calculated using the Weir equation [metabolic rate (kcal per day) = 1.44 (3.94 VO_2 + 1.11 VCO_2)] (71-73).

Doubly Labelled Water

Total daily energy expenditure during free-living conditions over 14 days (see Table 2) will be quantified using the criterion doubly labelled water (DLW) technique at pre-baseline, and at Day 1 3-month and 9-month appointments in a subsample of participants (n=24 total, 12 per group). Each participant will be asked if they would like to participate in doubly labelled water testing until the required sample is achieved. On each occasion, participants will be provided with a dose of isotope labelled water (10 atom% oxygen 18 (^{18}O) and 99.9 atom% deuterium (^{2}H)) with the dose based on body mass (1.35 g of DLW × body mass in kg). Participants will be asked to collect urine specimens daily over a 2-week period. Samples will be analysed by isotope ratio mass spectrometry (IRMS). Total daily energy expenditure (kJ) over the 2-week period will be divided by 14 to estimate mean total daily energy expenditure (74, 75).

Blood Pressure

At Day 1 baseline, 3-month and 9-month appointments, seated blood pressure will be recorded in a controlled environment using an automated sphygmomanometer and appropriately sized cuffs after a 5-minute quiet rest according to JNC 7 guidelines (76). The same arm will be used for all assessment visits with the appropriately sized cuff. Four consecutive readings will be recorded at ~2 min intervals with the mean of the last three measurements used for analysis.

Flash Glucose Monitoring

Flash glucose monitoring will be used to assess dynamic changes in glucose over a 2 week period at pre-baseline and 2 weeks before the end of both the weight loss and weight maintenance periods (FreeStyle Libre Flash Glucose Monitoring System) (77). Participants will wear a sensor on the back of their upper arm for up to 14 days and have a reader to scan the sensor every 6-8 hours. This system measures interstitial glucose concentrations and continuously stores measurement values every 15 minutes, which will provide information about postprandial glucose responses as well as changes in glucose regulation during weight loss. Average interstitial glucose and AUC (78) will be calculated and evaluated at each time point (see Table 2).

Dietary analysis

Adherence to energy-restricted diets will be assessed using 3 x 24-hour dietary recalls (via phone, at random times) during the weight loss and weight maintenance phases. Participants will also be asked to complete a 4-day weighed food record at pre-baseline, and 2 weeks before the end of both the weight loss and weight maintenance periods. Participants will be asked to record all foods and drinks consumed during this time and to record weights or estimate volumes using standard measures where possible and provide as much detail as possible about branded products. If required, we will lend participants a set of kitchen food scales.

Data will be collected on non-consecutive days and entered into Foodworks Nutritional Analysis Software version 8 (Xyris Software, Brisbane, QLD, Australia) for analysis of macronutrient and micronutrient intake as well as total energy intake.

Study Food Liking and Palpability Scores

A liking score for almonds and the carbohydrate-rich snack foods will be assessed using a Labelled Affect Magnitude Scale (79) and a food action rating scale (80) which will rate foods for overall liking, liking of textures and liking of flavours. These tests will occur at screening and at Day 1 9 months to determine any change following long-term consumption of the test foods.

Eating Behaviour, Mood and Personality

Eating behaviour, mood and personality will be assessed at Day 1 and Day 2 appointments at baseline, 3 months and 9 months (see Table 2). Change over time will be assessed using a series of validated questionnaires. Eating behaviour via; Power of Food (81), Food Craving Scale (82), Eating Attitude Test (83), Pickiness/Finickiness Questionnaire (84), EAT-26 (83), Control of Eating Questionnaire (85). Mood and personality via; Profile of Mood States (86), Perceived Stress Scale (87), Zung Self-Rating Scale (88), Eysenck Personality Questionnaire (89), and Brief Sensation Seeking Scale (90).

Quality of Life, Functional Mobility and Pain

The Timed Up and Go (TUG) test is a test of functional mobility. This test requires participants to be timed while getting up, walking 3 meters, turning, returning to the chair, and sitting down again (91). Previous studies in adults have reported the CV error was 6% for the TUG test (92). The short-form 36 (SF36) questionnaire will be used for assessing overall quality of life, and pain will be assessed with the SF36 bodily pain subscale as well as a VAS scale to rate the intensity of pain at each major chronic and/or acute pain site. The nature of pain (at each site) will be rated using a short-form of the McGill pain questionnaire (93). All pain measures have been shown to be reliable and valid in adults (94), with the psychometrics of the SF-36 specific to the Australian population (95, 96). Assessments will occur at Day 1 and Day 2 appoinments at baseline, 3 months and 9 months (see Table 2).

Faecal Microbiota

Stool samples will be collected at pre-baseline and 2 weeks before the end of the weight loss and weight maintenance phases using OMNIgene GUT DNA Stabilization Kits (DNA Genotek). DNA extraction will be performed using MoBio Powerlyzer Powersoil DNA Isolation Kit (MoBio Laboratories, Carlsbad, California)

as described previously (97). DNA concentration will be quantified fluorometrically with a Qubit dsDNA HS Assay kit (Life Technologies). Faecal microbial composition will be assessed by 16S rRNA amplicon sequencing (98). This analysis will include determination of Firmicutes:Bacteroidetes ratios and quantification of *Akkermansia muciniphila* abundance. A subsample of highest responders (n=10) and lowest responders (n=10) (based on weight regain) will also undergo more detailed analysis of samples from pre-baseline, end of weight loss and end of weight maintenance. Samples will be assessed for identification of responder/non-responder phylogenetic and functional traits using shotgun metagenomic sequencing (99).

Liver Health

The amount of fat stored within the liver will be assessed using ultrasound at Day 2 baseline, 3-month and 9-month appointments. Participants will have their livers imaged using a clinical ultrasound scanner (Philips iU22 Ultrasound imaging system, Bothell, WA, USA) equipped with a 5-1 MHz high-resolution curved array transducer (Model c5-1, Philips). The amount of fat stored within the liver will be assessed using three different methods. Firstly, the size of the liver will be determined using the technique by Childs et al (100). Three linear measurements of the liver will be taken and then volume calculated by using the following equation: liver volume (cm^3) = 343.71 + [0.84 × ABC], where ABC is the product of the three linear measurements (100). Secondly, a visual assessment of fat accumulation will be evaluated according to the technique by Ballestri et al, adapted to liver images (101). A fatty liver indicator score ranging from 0 to 9 will be calculated using three indicators: (1) presence or absence of liver-kidney contrast; (2) assessment of beam penetration and (3) level of vessel wall blurring. Each measure will be graded as normal (score of 0), mild (score of 1), moderate (score of 2), or severe (score of 3) (101). Finally, quantification of liver fibrosis, a consequence of fatty liver, will be performed using shear wave elastography. The ultrasound machine emits a low-frequency pulse and software inbuilt into the ultrasound machine indicates the density of the liver tissue. The more fibrotic the tissue, the denser the tissue and the higher the reading (102).

Data Management

All participants will be given a unique code, which will be used to identify their electronic and paper-based data and biological samples. A password-protected computer database, accessible by the researchers only, will store participant identifiers (e.g., name, email address, phone number) and their associated code. Paper-based data will be stored securely at the University of South Australia for 15 years, after which it may be destroyed. Paper case report forms will be used to collect data at all clinic visits. Data will be entered twice into two separate password-protected Excel data files. Before analysis, data will be compared between files to ensure it has been correctly recorded. Biological samples will be stored in a swipe card secured −80°C freezer, with an alarm system that alerts a staff member if the temperature rises above a predetermined temperature. Samples will be stored for up to 15 years from collection date and disposed of accordingly after that time.

Protocol deviations

Deviations from the proposed protocol will be communicated via an update of the Australian and New Zealand Clinical Trial Registry and also through a letter to the editor of this journal.

Adverse Events

Adverse events will be recorded in the case report form and will be reported to the University of South Australia Human Research Ethics Committee. Adverse events that lead to withdrawals will be reported in future publications. We do not intend to formally analyse adverse events.

Statistical analysis plan

Statistical analysis will be performed using SPSS for Windows 24.0 (SPSS, Chicago, IL, USA). Data will be tested for normality, and where possible non-normally distributed data will be log transformed prior to analysis. The effects of diet treatment on the dependent measures over time will be analysed using random effects mixed models, with treatment (AED or NFD) and time (baseline, end of weight loss, and end of weight maintenance) being the between and within factors respectively in the

analysis. Both intention-to-treat (ITT) and per-protocol analyses (for those who achieve a minimum of 80% compliance with test food consumption) will be completed. Where main effects are identified, Bonferroni post hoc tests will be performed to identify significant differences between means (P set at <0·05). Whilst the ITT analysis will be the main analysis, the per-protocol analysis will allow us to decipher that the effects are due to participants being compliant with consuming test foods. We will also run sub-analyses for both the ITT and per-protocol analyses which will stratify participants according to weight loss during the weight loss phase to determine whether there is an interaction between dietary treatment and weight loss in terms of effects on the various outcomes. This will allow for determination of whether almonds, compared with control, provide greater improvements in outcomes for any given level of weight loss.

Data Access

There are no contractual agreements that require the data from this trial to be shared.

Ethics and Dissemination

Ethics approval was obtained from the University of South Australia Human Research Ethics Committee (201436).

Participants will receive a copy of their individual results (with the exception of liver scans, faecal sample testing, resting energy expenditure, doubly labelled water and some blood analysis results) as well as a summary of the study findings. Participants who complete all aspects of the study will receive an honorarium of $400 to compensate for their time and travel expenses. The subsample of participants who also participate in doubly labelled water assessments will be provided with an additional $150 honorarium. Findings of the study will be disseminated at scientific conferences and in published papers. Further, where appropriate outcomes will be made available to the public via media releases.

Trial Registration

The protocol for this study was registered with the Australian New Zealand Clinical Trials Registry (ACTRN12618001861246).

DISCUSSION

Nut consumption in many countries is low, and it has been suggested that people may avoid eating nuts as they may perceive that the high-fat content will lead to weight gain (12, 13, 15, 16). However, various mechanisms have been hypothesized regarding the association between nut consumption and weight loss success. Nuts are rich in protein, fibre and monounsaturated fat, macronutrients known to have a positive effect on appetite control (25). The high unsaturated fat content and protein in nuts may lead to an increase in resting energy expenditure and diet-induced thermogenesis (31). Additionally, the structure of the lipid-storing granules and high fibre content of nuts, as well as incomplete mastication may cause reduced fat absorption resulting in a loss of available energy (31). Despite this, clinical trials evaluating the effects of nut consumption compared to a nut-free diet on adiposity have reported mixed findings, with some studies reporting greater weight loss (17, 18) or improved body composition (19, 20), and others reporting no weight change (1, 5, 9), suggesting further investigation is required.

Strengths

There are a number of novel features in this study design. To our knowledge, this will be the first trial to assess whether the inclusion of 15% of energy from almonds vs. 15% of energy from carbohydrate-rich snack foods in an otherwise nut-free diet will improve weight loss and limit weight regain. As such, this work has the potential to expand the current understanding of how regular consumption of almonds can aid weight loss and weight maintenance, while also providing beneficial cardiometabolic, liver and gut health effects. Furthermore, the metagenomic analysis that will be performed will be a substantial advance on our current understanding of the impact of almonds on the gut microbiome (previously limited to amplicon sequencing approaches) (103).

Limitations

One of the main challenges with any dietary intervention study is recruiting participants and keeping them motivated. We have previously successfully recruited large numbers of participants for similar intervention trials and plan to support participants with regular appointments with the dietitian, as well as the provision of test foods and simple food checklists to assist with compliance. A potential limitation of this study is that it will only be feasible to follow participants for 6 months after initial weight loss, although data from our recent pilot study suggests that this will be sufficient to observe differential changes in weight regain due to the rapidity of weight regain once intensive dietary support is removed (Wilson AL et al. Nudging Weight Loss Maintenance in Adults with Type-2 Diabetes: A Pilot Intervention).

This 9-month randomised controlled trial will add to the evidence base strategies to facilitate weight loss and prevent regain, potentially leading to recommendations to frequently substitute energy-dense snacks that lack nutritional value with nuts, which, in turn, could lead to improved weight loss outcomes and facilitate beneficial dietary habits (13, 104).

Author contributions: A.M.C, J.D.B, A.M.H, S.Y.T, G.B.R : were co-applicants on the grant application and as such were involved with the original design. A.M.C: was the lead applicant and is the principal investigator for the study. A.M.C, J.D.B, A.M.H, S.C, C.Y: are involved with study coordination and responsible for the day to day running of the trial, recruitment and sample collection. All authors (S.C, A.M.H, C.Y, J.D.B, S.Y.T, G.B.R, J.C, M.M, K.L, S.W, T.R.S, F.F, A.P.H and A.M.C): contributed to method development and the writing and development of the protocol paper and all authors will have responsibility for analysis, statistical interpretation of outcomes and preparation of manuscripts for publication post-study completion.

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Conflicts of interest: This study was funded by the Almond Board of California. A.M.C has consulted for Nuts for Life (an initiative of the Australian Tree Nut Industry).

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Figure Legend

Table 1. Eligibility Criteria

Table 2. Outcome Measures at Each Time Point

Table 3. Macronutrient Composition of Test Foods

Table 4. Blood and Faecal Analysis

Figure 1. Study Timeline

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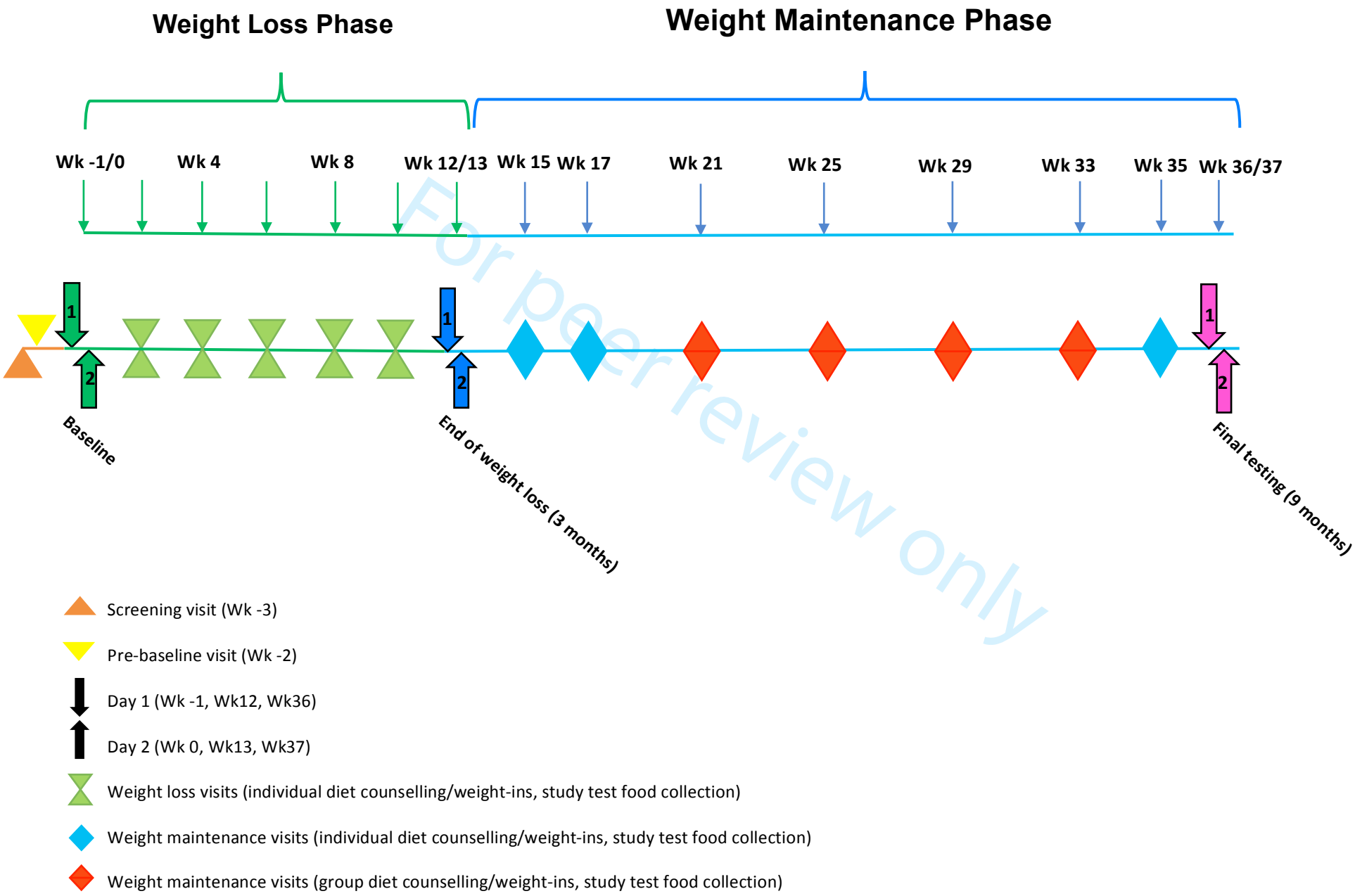
Figure 1: Study Timeline



SPIRIT
STANDARD PROTOCOL ITEMS: RECOMMENDATIONS FOR INTERVENTIONAL TRIALS

SPIRIT 2013 Checklist: Recommended items to address in a clinical trial protocol and related documents*

Section/item	Item No	Description	Addressed on page number
Administrative information			
Title	1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym	1
Trial registration	2a	Trial identifier and registry name. If not yet registered, name of intended registry	1
	2b	All items from the World Health Organization Trial Registration Data Set	N/A
Protocol version	3	Date and version identifier	1
Funding	4	Sources and types of financial, material, and other support	28
Roles and responsibilities	5a	Names, affiliations, and roles of protocol contributors	1
	5b	Name and contact information for the trial sponsor	1
	5c	Role of study sponsor and funders, if any, in study design; collection, management, analysis, and interpretation of data; writing of the report; and the decision to submit the report for publication, including whether they will have ultimate authority over any of these activities	28
	5d	Composition, roles, and responsibilities of the coordinating centre, steering committee, endpoint adjudication committee, data management team, and other individuals or groups overseeing the trial, if applicable (see Item 21a for data monitoring committee)	28

Introduction			
Background and rationale	6a	Description of research question and justification for undertaking the trial, including summary of relevant studies (published and unpublished) examining benefits and harms for each intervention	3-5
	6b	Explanation for choice of comparators	12
Objectives	7	Specific objectives or hypotheses	4-5
Trial design	8	Description of trial design including type of trial (eg, parallel group, crossover, factorial, single group), allocation ratio, and framework (eg, superiority, equivalence, noninferiority, exploratory)	5-7
Methods: Participants, interventions, and outcomes			
Study setting	9	Description of study settings (eg, community clinic, academic hospital) and list of countries where data will be collected. Reference to where list of study sites can be obtained	5
Eligibility criteria	10	Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and individuals who will perform the interventions (eg, surgeons, psychotherapists)	6
Interventions	11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered	12-13
	11b	Criteria for discontinuing or modifying allocated interventions for a given trial participant (eg, drug dose change in response to harms, participant request, or improving/worsening disease)	N/A
	11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return, laboratory tests)	12
	11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial	N/A
Outcomes	12	Primary, secondary, and other outcomes, including the specific measurement variable (eg, systolic blood pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg, median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen efficacy and harm outcomes is strongly recommended	13-24
Participant timeline	13	Time schedule of enrolment, interventions (including any run-ins and washouts), assessments, and visits for participants. A schematic diagram is highly recommended (see Figure)	9-11

Sample size	14	Estimated number of participants needed to achieve study objectives and how it was determined, including clinical and statistical assumptions supporting any sample size calculations	7
Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size	6-7
Methods: Assignment of interventions (for controlled trials)			
Allocation:			
Sequence generation	16a	Method of generating the allocation sequence (eg, computer-generated random numbers), and list of any factors for stratification. To reduce predictability of a random sequence, details of any planned restriction (eg, blocking) should be provided in a separate document that is unavailable to those who enrol participants or assign interventions	7
Allocation concealment mechanism	16b	Mechanism of implementing the allocation sequence (eg, central telephone; sequentially numbered, opaque, sealed envelopes), describing any steps to conceal the sequence until interventions are assigned	7
Implementation	16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions	7
Blinding (masking)	17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how	7
	17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial	7
Methods: Data collection, management, and analysis			
Data collection methods	18a	Plans for assessment and collection of outcome, baseline, and other trial data, including any related processes to promote data quality (eg, duplicate measurements, training of assessors) and a description of study instruments (eg, questionnaires, laboratory tests) along with their reliability and validity, if known. Reference to where data collection forms can be found, if not in the protocol	9-11, 13-24
	18b	Plans to promote participant retention and complete follow-up, including list of any outcome data to be collected for participants who discontinue or deviate from intervention protocols	6

Data management	19	Plans for data entry, coding, security, and storage, including any related processes to promote data quality (eg, double data entry; range checks for data values). Reference to where details of data management procedures can be found, if not in the protocol	24-25
Statistical methods	20a	Statistical methods for analysing primary and secondary outcomes. Reference to where other details of the statistical analysis plan can be found, if not in the protocol	25
	20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	25
	20c	Definition of analysis population relating to protocol non-adherence (eg, as randomised analysis), and any statistical methods to handle missing data (eg, multiple imputation)	25
Methods: Monitoring			
Data monitoring	21a	Composition of data monitoring committee (DMC); summary of its role and reporting structure; statement of whether it is independent from the sponsor and competing interests; and reference to where further details about its charter can be found, if not in the protocol. Alternatively, an explanation of why a DMC is not needed	N/A
	21b	Description of any interim analyses and stopping guidelines, including who will have access to these interim results and make the final decision to terminate the trial	N/A
Harms	22	Plans for collecting, assessing, reporting, and managing solicited and spontaneously reported adverse events and other unintended effects of trial interventions or trial conduct	25
Auditing	23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor	N/A
Ethics and dissemination			
Research ethics approval	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval	2
Protocol amendments	25	Plans for communicating important protocol modifications (eg, changes to eligibility criteria, outcomes, analyses) to relevant parties (eg, investigators, REC/IRBs, trial participants, trial registries, journals, regulators)	2 & 25

Consent or assent	26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)	7
	26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	N/A
Confidentiality	27	How personal information about potential and enrolled participants will be collected, shared, and maintained in order to protect confidentiality before, during, and after the trial	24
Declaration of interests	28	Financial and other competing interests for principal investigators for the overall trial and each study site	28
Access to data	29	Statement of who will have access to the final trial dataset, and disclosure of contractual agreements that limit such access for investigators	24
Ancillary and post-trial care	30	Provisions, if any, for ancillary and post-trial care, and for compensation to those who suffer harm from trial participation	N/A
Dissemination policy	31a	Plans for investigators and sponsor to communicate trial results to participants, healthcare professionals, the public, and other relevant groups (eg, via publication, reporting in results databases, or other data sharing arrangements), including any publication restrictions	26
	31b	Authorship eligibility guidelines and any intended use of professional writers	28
	31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	26
Appendices			
Informed consent materials	32	Model consent form and other related documentation given to participants and authorised surrogates	Yes, sent to editor
Biological specimens	33	Plans for collection, laboratory evaluation, and storage of biological specimens for genetic or molecular analysis in the current trial and for future use in ancillary studies, if applicable	24

*It is strongly recommended that this checklist be read in conjunction with the SPIRIT 2013 Explanation & Elaboration for important clarification on the items. Amendments to the protocol should be tracked and dated. The SPIRIT checklist is copyrighted by the SPIRIT Group under the Creative Commons "Attribution-NonCommercial-NoDerivs 3.0 Unported" license.